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Given the above, you should not use this document as a tool, guide, or manual for the preparation of applications or submissions to FDA. Instead, all interested persons should refer to the Federal Food, Drug, and Cosmetic Act, and its implementing regulations, as well as guidance documents prepared by FDA, for information on FDA's tobacco authorities and regulatory framework. FDA also regularly posts additional resources for applicants, such as webinars and application tips, on CTP's website and social media.

U.S. FOOD & DRUG ADMINISTRATION

Memorandum

Date: June 3, 2024

To: File

From: Toyin Ajao, PhD
Biologist
Division of Nonclinical Science

Digitally signed by Toyin T. Ajao -S
Date: 2024.06.03 14:38:51 -04'00'

Chad Brocker, PhD, DABT
Supervisory Toxicologist
Division of Nonclinical Science

Digitally signed by Chad N. Brocker -S
Date: 2024.06.03 14:42:52 -04'00'

Mamata De, PhD
Pharmacologist
Division of Nonclinical Science

Digitally signed by Mamata De -S
Date: 2024.06.03 14:47:19 -04'00'

Jonathan Fallica, PhD
Supervisory Toxicologist
Division of Nonclinical Science

Digitally signed by Jonathan Fallica -S
Date: 2024.06.03 14:48:09 -04'00'

Matthew Hartog, PhD
Toxicologist
Division of Nonclinical Science

Digitally signed by Matthew A. Hartog -S
Date: 2024.06.03 14:50:10 -04'00'

Anwar Husain, MD, PhD
Toxicologist
Division of Nonclinical Science

Digitally signed by Anwar Husain -S
Date: 2024.06.03 14:52:55 -04'00'

Mary Irwin, PhD
Supervisory Pharmacologist
Division of Nonclinical Science

Digitally signed by Mary Irwin -S
Date: 2024.06.03 14:56:34
-04'00'

Kristen Wurcel, PhD
Supervisory Pharmacologist
Division of Nonclinical Science

Digitally signed by Kristen D. Wurcel -S
Date: 2024.06.03 14:59:02 -04'00'

Through: Luis G. Valerio, Jr., PhD
Associate Director

Digitally signed by Luis G. Valerio -S
Date: 2024.06.03 15:24:37 -04'00'

Division of Nonclinical Science

Berran Yucesoy, PhD
Deputy Director
Division of Nonclinical Science

Digitally signed by Berran Yucesoy -S
Date: 2024.06.03 15:27:30 -04'00'

Hans Rosenfeldt, PhD
Director
Division of Nonclinical Science

Digitally signed by Hans M. Rosenfeldt -S
Date: 2024.06.03 16:32:21 -04'00'

Subject: Genotoxicity Hazard Identification and Carcinogenicity Tiering of Constituents in ENDS Premarket Tobacco Product Applications

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Introduction

Premarket Tobacco Product Applications (PMTAs) are submitted for any new tobacco product seeking an FDA marketing order under section 910(b) of the Federal Food, Drug, and Cosmetic (FD&C) Act. A PMTA is required to provide sufficient scientific evidence demonstrating that marketing of the new product is appropriate for the protection of public health (APPH). Scientific data must address, among other things, any health risks and benefits of the new product to the US population as a whole. This includes people who use the new product, as well as nonusers. To address this requirement, PMTAs must include full reports of all available information known to, or which should reasonably be known to, the applicant concerning studies that show any health risks associated with the new product and whether the new product presents less risk than other marketed tobacco products. PMTAs should also include a complete listing of components included in the new product as detailed in Section 910(b)(1)(B) of the FD&C Act and § 1114.7(i)(1) of the PMTA rule, which require a PMTA for a new tobacco product to contain a full statement of the components, ingredients, additives, and properties. The product components, ingredients, additives, and properties have a direct impact on the toxicity of the product by influencing the total yield and delivery of harmful and potentially harmful constituents (HPHCs) and other potentially toxic constituents to the product user. In general, toxicological data submitted with a PMTA includes relevant non-clinical studies intended to address any potential toxicological risks associated with the new product (e.g., cytotoxicity, genotoxicity).

The final PMTA rule[1] emphasizes that an evaluation of genotoxicity and carcinogenicity is important in PMTAs and describes details regarding the toxicological profile of new tobacco products. Specifically, under 21 CFR § 1114.7(k)(1)(i)(B), a PMTA must contain:

"The toxicological profile of the new tobacco product related to the route of administration, including the genotoxicity, carcinogenicity, reproductive toxicity, immunotoxicity, acute toxicity, and repeat dose (chronic) toxicity of the new tobacco product relative to other tobacco products. The toxicological profile

[1]Premarket Tobacco Product Applications and Recordkeeping Requirements October 2021. 86 Fed. Reg. at 55300 - https://www.federalregister.gov/documents/2021/10/05/2021-21011/premarket-tobacco-product-applications-and-recordkeeping-requirements

also includes information on the toxicity of the ingredients, additives, and HPHCs, relative to the route of administration and the range of potential levels of exposure resulting from the use of, or exposure to, the new tobacco product, including studies which discuss the toxicological effects of any leachables and extractables that can appear from the container closure system and the ingredient mixture, such as additive or synergistic effects[.]"

In 2023, FDA issued a guidance for industry (USFDA, 2023) for PMTA Electronic Nicotine Delivery System (ENDS) submissions which recommends "*providing a full assessment of the toxicological and pharmacological profile*" of a new tobacco product using:

- "*Toxicology data from the literature (i.e., all relevant publications);*
- *Analysis of constituents, including HPHCs and other toxicants, under both intense and non-intense use conditions;*
- *In vitro toxicology studies (e.g., genotoxicity studies, cytotoxicity studies);*
- *Computational modeling of the toxicants in the product (to estimate the toxicity of the product); and*
- *In vivo toxicology studies (to address unique toxicology issues that cannot be addressed by alternative approaches)*."

The purpose of this memorandum is to describe the process CTP DNCS toxicology reviewers (reviewers) should use for genotoxicity hazard identification and carcinogenicity tiering of constituents during ENDS PMTA review. This process is intended to support reviewers' understanding of their role in evaluating the information submitted in an ENDS PMTA. FDA routinely creates standardized processes to improve the consistency and efficiency of review processes. The scientific evaluation process outlined in this document creates a standardized approach for toxicology reviewers to describe carcinogenic or potential carcinogenic hazards of constituents and identify, if appropriate, what types of evidence may clarify such hazards for those constituents. Genotoxicity hazard identification and carcinogenicity tiering of constituents is part of the cancer risk evaluation, which is one aspect of toxicology PMTA review. Toxicology's cancer risk evaluation can be integrated along with findings from other review disciplines by the technical project lead (TPL) in overall decision-making regarding whether the marketing of new products under review is APPH. This memorandum also outlines specific issues that DNCS has encountered in the evaluation of genotoxicity assays in ENDS PMTAs. This memorandum applies to ENDS; however, information within this memorandum may also be applicable to other types of new tobacco products submitted through the PMTA pathway (e.g., combusted tobacco products, smokeless tobacco products, oral nicotine products, non-tobacco nicotine products) and could potentially be used in reviews for other product categories that cite a rationale for why the content in this memorandum is relevant to the evaluation of the reviewed products.

As part of an APPH determination, tobacco products must undergo an evaluation of their genotoxic and carcinogenic potential before a marketing order can be granted. Current scientific literature demonstrates that ENDS generally have fewer and lower yields of HPHCs than combusted cigarettes. Risk comparisons between combusted cigarettes and ENDS based upon HPHCs is a useful initial assessment, however, this approach does not consider other toxic constituents present in ENDS that are not on the established HPHC list in the overall risk evaluation. Genotoxic constituents in ENDS can

originate from the direct addition of ingredients to the product, the migration of chemicals from container or packaging components (i.e., leachables), as well as through the degradation and/or pyrolysis of these constituents during combustion or aerosolization. Our experience from review of PMTAs indicates that other constituents (e.g., e-liquid ingredients, leachables from the container or packaging components), along with those on the established HPHC list, have the potential to confer substantial risk for adverse health effects, including cancer risk, for ENDS. The review of PMTAs to date has also revealed many concerns and challenges associated with how genotoxicity data from ENDS are evaluated and interpreted by toxicology reviewers and applicants. Moreover, the experience that DNCS has gathered from PMTA review to date now allows for reviewers to utilize a more quantitative evaluation of the carcinogenic risk from HPHCs and other constituents with identified hazards in ENDS. As a result, our scientific approach for evaluating and interpreting the genotoxicity of ENDS has developed to include a quantitative assessment of the excess lifetime cancer risk (ELCR) posed by the product. Following a hazard identification process, this quantitative assessment should be performed to evaluate the genotoxic hazards posed by the product's individual constituents.

Several technical issues and concerns regarding genotoxicity hazard identification assays are described herein. These technical issues and concerns can limit, as a reviewer, your ability to draw reliable conclusions from genotoxicity studies using ENDS e-liquids, aerosols, and aerosol condensates. Most of the concerns arise from the fact that these common ENDS test articles are complex mixtures and that standard hazard identification analyses are not specifically designed for such test articles. The assessment of whole mixtures in these assays is not impossible, but the results can be much harder to evaluate and interpret than single molecular entity test articles for a myriad of technical reasons. While whole mixtures (e.g., e-liquid, aerosol) may be analytically characterized to quantify individual constituents for an exposure assessment, using whole mixtures to evaluate the downstream toxic responses within an experimental system for hazard identification purposes is readily confounded for these ENDS mixtures by the presence of multiple constituents that have known toxic effects, as well as similar or complementary mechanisms of toxicity, biological targets, and detoxification pathways. These complex interactions make it difficult to ascertain whether a particular constituent in a mixture is a genotoxic hazard because positive genotoxic responses from the mixture could be explained by the presence of other constituents in the mixture. Moreover, standard genotoxicity studies do not provide relative assessments – they cannot determine whether a test article is "more genotoxic" than another; the only output from standard genotoxicity assessments is whether a test article response is positive, negative, or equivocal for genotoxicity.

As a solution to minimize confounding effects within hazard identification assays, reviewers should evaluate these complex mixtures using a component-based approach. A component-based approach involves the assessment of each individual constituent present in the mixture. Related regulatory documents recommend using a component-based approach for genotoxicity testing of fully defined or characterized mixtures (EFSA, 2011; EFSA et al., 2019; OECD, 2018; USEPA, 2007; WHO, 2009). In such an approach, hazard identification results from the genotoxicity assessment of individual ENDS constituents and a tiered weight of evidence (WOE) approach can be used to evaluate and classify all ENDS constituents (e.g., HPHCs, ingredients, and leachables). Under this approach, analytically quantified constituent yields provided to Toxicology from Chemistry can be used along with constituent

tiering determinations to inform a cancer risk assessment that allows for reviewers to consistently perform a comparison of cumulative estimated cancer risk between marketed tobacco products.

A consistent approach to hazard identification of all potentially genotoxic constituents (e.g., HPHCs, ingredients, leachables) is critical to assessing product risk from a toxicological perspective. Toxicology reviewers should follow the genotoxicity hazard identification principles and carcinogenicity tiering of constituents, as described in this memorandum, followed by an evaluation to assess the cumulative estimated cancer risk posed by a new product, as detailed in a separate DNCS memorandum (see Memorandum: Calculating Excess Lifetime Cancer Risk in Tobacco Product Applications, June 3, 2024). Based on our experience reviewing PMTAs, the new reviewer workflow outlined in this memorandum is intended to be more informative and better assess toxicological risks associated with ENDS, which lack human epidemiological data on long-term health risks.

Background

Tobacco products contain numerous chemical constituents that may pose a genotoxicity concern and contribute to cancer risk. For the purposes of this memorandum, reviewers are to consider genotoxicants as chemicals that induce adverse effects (e.g., DNA damage) on genetic components through a variety of mechanisms. The occurrence of DNA damage is a critical initial step in the production of a mutation following exposure to a mutagenic constituent (IARC, 2019b). Data in the literature indicate that 80 - 90% of carcinogens have a genotoxic mode of action (Bartsch et al., 1989) and a comprehensive analysis demonstrated that over 90% of recognized IARC Group 1 chemical carcinogens are mutagenic (Waters et al., 1999). Therefore, genotoxicity is a critical mechanism for carcinogenesis.

In general, early conclusions from PMTA toxicology reviews were that ENDS were unlikely to raise genotoxicity concerns compared to combusted cigarettes. This was primarily based on our observations that ENDS PMTAs commonly showed relatively large reductions in HPHCs, many of which are known genotoxicants and carcinogens, and that ENDS test articles (e.g., aerosols and aerosol condensates) reported negative results from in vitro genotoxicity assays.

From a toxicology perspective, ENDS users are potentially exposed to genotoxic constituents present in the inhaled aerosol arising from three distinct sources:

- Thermal degradation or reaction products of e-liquid constituents (e.g., ingredients and leachables), or chemical adducts of e-liquid constituents, that transfer to the aerosol
- E-liquid ingredients that transfer directly to the aerosol
- Leachables that migrate from ENDS container closure systems and components into the e-liquid and transfer to the aerosol

Of these three sources of genotoxicants, many genotoxic thermal degradation products associated with ENDS are found on FDA's HPHC list established in 2012. This preliminary list of 93 HPHCs and the proposed list of 19 additional HPHCs collectively identify chemicals linked to the five most serious health effects of tobacco product use (i.e., cancer, cardiovascular disease, respiratory, reproductive toxicities,

and addiction) (USFDA, 2012, 2019). These constituents are a key toxicological concern as they contribute to the adverse health effects resulting from use of traditional tobacco products, such as combusted cigarettes and smokeless tobacco products. The thermal degradation of e-liquid constituents during aerosolization is known to generate many genotoxicants. For example, the oxidation of the e-liquid ingredients propylene glycol and glycerol is a source of the genotoxic carbonyl compounds formaldehyde and acetaldehyde that have been detected and quantified in ENDS aerosols (Farsalinos et al., 2017). HPHC yields in ENDS aerosols are quantitatively measured using analytical chemistry techniques. These data are submitted with PMTAs and toxicology reviewers should use the data to perform HPHC toxicity and exposure assessments.

In addition to genotoxic constituents formed during the aerosolization process, ingredients found in the e-liquid can also directly transfer into the ENDS aerosol. E-liquids typically include nicotine (either as freebase or nicotine salts), propylene glycol, vegetable glycerin, and flavoring ingredients. A survey of 16,839 e-liquids found that, on average, 10 (range of 3 - 18 across flavor categories) flavoring ingredients are added to a single e-liquid and that, on average, 63% of the total number of ingredients in e-liquids are flavoring ingredients (Krusemann et al., 2021). A study by Behar et al (Behar et al., 2018), showed twelve of the most common e-liquid flavor ingredients, including cinnamaldehyde, menthol, benzyl alcohol, vanillin, eugenol, p-anisaldehyde, ethyl cinnamate, maltol, ethyl maltol, triacetin, benzaldehyde, and menthone, are often present in concentrations above 1 mg/mL in e-liquids. It was also found that these constituents can transfer efficiently into the ENDS aerosol (e.g., mean transfer efficiency ≈ 98% across all compounds) (Behar et al., 2018). As such, the inherent toxicity, and more specifically the genotoxicity, of e-liquid constituents is a concern. For instance, many flavor ingredients contain aldehyde functional groups that potentially form toxic chemical adducts and are transferred to the aerosol (Jabba et al., 2020).

Leachables are another potential source of genotoxic hazards in ENDS that reviewers should consider in their evaluation. Measuring the toxicity of, and exposure to, leachables present in e-liquids is a priority for CTP. As described in Norwood et al. (Norwood et al., 2008), leachables are organic or inorganic chemicals that migrate from container closure system (CCS) components (i.e., coil, wicking material, glass or plastic vial container or cartridge, etc.) into the finished product. Published literature and ENDS PMTAs have shown that toxic chemicals such as cadmium, chromium, lead, nickel, chloroform, dichlorobenzene, bisphenol A, phthalates, parabens, and organophosphate flame retardants can leach into e-liquids of ENDS (Gray et al., 2022; Halstead et al., 2020; Wei et al., 2020). ENDS users may be exposed to leachables during normal, routine use of the product, making leachables, in addition to other constituents, a potential toxicological concern for toxicology reviewers to consider in their reviews.

While some ENDS constituents have a wealth of information regarding their toxicities, other ENDS constituents are data-limited (i.e., experimental toxicity data is either lacking or inadequate to inform a toxicological evaluation), include equivocal studies (i.e., a study is neither clearly positive nor clearly negative), or have conflicting hazard outcomes (i.e., both positive and negative study results) making it difficult to confidently assess genotoxicity. There are constituents of toxicological concern in ENDS that are not included on the established list of HPHCs, and the genotoxicity hazards of such ingredients, leachables, and other constituents (e.g., ingredient and leachable reaction products) identified in ENDS are often unknown, limited, or inconclusive. To evaluate potential carcinogenicity of ENDS, DNCS

determines if these constituents are reasonably expected to be genotoxic, mutagenic, or carcinogenic. As part of this approach, reviewers should evaluate constituents for genotoxicity hazards and cancer risk. This evaluation should consider a combination of applicant submitted and publicly available hazard identification assays (in silico, in vitro, or in vivo), scientific literature, and toxicological database searches. In addition to peer-reviewed literature, databases from relevant agencies including, but not limited to, the European Food Safety Authority (EFSA), the Joint FAO/WHO Expert Committee on Food Additives (JECFA), National Toxicology Program (NTP), United States Environmental Protection Agency (EPA), Agency for Toxic Substances and Disease Registry (ATSDR), National Institute for Occupational Safety and Health (NIOSH), International Agency for Research on Cancer (IARC), and European Chemicals Agency (ECHA) may be used to screen compounds for available genotoxicity, mutagenicity, or carcinogenicity data. It is important to note that the hazard identification process used by CTP toxicology reviewers is consistent with standard approaches used by other regulatory agencies, however, the WOE risk characterization procedure outlined in subsequent sections is specific to tobacco products.

The PMTA rule[2] requires applicants to provide supporting data that addresses genotoxicity hazards associated with the new products in their PMTAs. Commonly, applicants include standard in vitro or in vivo assays to assess the genotoxicity or mutagenicity of ENDS e-liquids, aerosols, or aerosol condensates from their new products relative to comparison products (e.g., other ENDS and combusted cigarettes). It is important for toxicology reviewers to note that there are currently no validated approaches available to perform relative genotoxicity comparisons using standard hazard identification assays. In other words, genotoxicity assay results for hazard identification cannot discern if one test article is more, or less, genotoxic than another. Standard genotoxicity assays are qualitatively represented as “positive” (i.e., detected), “negative” (i.e., not detected), or “equivocal” (i.e., the data set does not allow a conclusion of positive or negative) on whether a test article is genotoxic via a specific toxicological mechanism and under the specific conditions of the assay used. As such, genotoxicity assays for hazard identification are used as indicators of whether a genotoxicity hazard, such as mutagenicity (i.e., Ames assay) or chromosomal damage (i.e., in vitro micronucleus [MN] assay), is present for a particular test article (i.e., e-liquid, aerosol, or aerosol condensate). This genotoxicity information is used in the assessment of the test article’s carcinogenic potential. Importantly, current international standards for genotoxicity assays (e.g., Organization for Economic Cooperation and Development [OECD] test guidelines) do not provide information regarding the evaluation of the magnitude of response (e.g., “weakly positive”), but rather focus on the clarity of result (i.e., clear positive, clear negative, or otherwise equivocal).

As noted previously, in practice, the presence of known toxic constituent(s) in a mixture generally prevents a hazard identification assay from providing useful information regarding the other components of the mixture. Because no carcinogenicity studies of ENDS mixtures are anticipated and because genetic toxicology studies cannot, at this time, provide a relative genotoxic assessment between products, assessing the hazard of individual components within a mixture is necessary for further evaluation of carcinogenic risk within standard risk assessment procedures. To address this challenge, a component-based approach can be used to evaluate the hazards associated with the

[2]Premarket Tobacco Product Applications and Recordkeeping Requirements October 2021. 86 Fed. Reg. at 55300 - https://www.federalregister.gov/documents/2021/10/05/2021-21011/premarket-tobacco-product-applications-and-recordkeeping-requirements

individual constituents of the complex mixture. When genotoxicity hazards are identified in tobacco products, a subsequent risk assessment is needed to determine the cumulative risk of the new product to provide information to the TPL to include as part of their consideration of whether marketing of the new product is APPH. In PMTAs for ENDS, applicant-provided primary studies are generally offered by applicants to justify the purported absence of genotoxicity concerns. But these new products often contain known genotoxicants and ingredients with unknown or limited toxicological information. From our experience with PMTA review, DNCS has identified several concerns related to genotoxicity studies submitted within applications, specifically those studies generated using mixtures such as ENDS e--liquids, aerosols, or aerosol condensates, which limit their utility in genotoxicity assessments. Importantly, these concerns affect our overall confidence in hazard identification from studies using these test articles and are discussed below.

Genotoxicity Assays for Hazard Identification of ENDS

Hazard identification (including identification of cancer and non-cancer hazards) is the first step in a reviewer's risk assessment and should be used in the initial evaluation of new products submitted through the PMTA pathway to assess the toxicological profile of products under review. Examples specific to genetic toxicology include occurrence of chromosomal damage, mutagenicity, and cytotoxicity. Accurate identification of hazards is crucial for a relevant risk assessment and determination of toxicological profile necessary for the review of PMTA applications. Experience garnered from PMTA review has identified several issues that limit our ability to confidently draw reliable conclusions from genotoxicity assays commonly used for hazard identification by applicants in PMTAs. Specifically, OS has significant concerns about the utility of using whole, complex mixtures that contain known genotoxicants or carcinogens from ENDS, such as e-liquids, aerosols, or aerosol condensates, as the test article in genotoxicity hazard identification assays.

ENDS e-liquids, aerosols, or aerosol condensates are complex mixtures that contain numerous constituents. E-liquid ingredients and leachables, along with other constituents, may be known genotoxicants or have unknown or limited toxicological information. Importantly, these constituents can efficiently transfer to the aerosol. Aerosolization and thermal degradation of e-liquid ingredients generates additional genotoxicants. The resulting ENDS aerosol may contain several known genotoxicants or potentially genotoxic compounds at differing concentrations. To further complicate genotoxicity testing, e-liquids and e-liquid aerosols may also contain potential co-carcinogens (i.e., tumor promoters) such as nicotine (Lee et al., 2018).

The use of ENDS aerosol or aerosol condensate as a test article in genotoxicity assays for hazard identification introduces genotoxic carcinogens such as acetaldehyde and formaldehyde to the assay. As such, the presence of known genotoxicants is a confounding factor as to the meaningfulness of related conclusions. For example, the presence of known genotoxic aldehydes in the aerosol is sufficient to conclude the whole mixture as genotoxic. Importantly, known genotoxic constituents in the mixture potentially mask genotoxic effects of other data-limited constituents thereby limiting the utility of the test to evaluate these data-limited constituents. Furthermore, an aerosol that contains multiple genotoxicants or tumor promoters may result in synergistic or additive effects on carcinogenesis. Interactions between genotoxicants may also produce antagonistic effects on carcinogenesis through a variety of mechanisms (e.g., increased cytotoxicity and DNA damage, cell cycle progression blockage,

gene expression changes). Given these potential interactions and the influence of genotoxic HPHCs common in ENDS aerosols, it is unlikely that standard in vitro or in vivo studies (e.g., Ames assay, in vitro MN assay, in vivo MN assay) conducted with ENDS aerosols or aerosol condensates can discern toxicological hazards posed by individual constituents of the e-liquid. A positive hazard identification in this situation provides little information on what constituents are driving the genotoxic risk of the new product. More importantly, a positive hazard identification using an e-liquid, aerosol, or aerosol condensate does not inform or contribute meaningful information to a subsequent cancer risk assessment that would allow for a comparison between tobacco products.

In a complex mixture, the genotoxic effects of a constituent in the ENDS test article (e.g., e-liquid, aerosol, or aerosol condensate) may be masked by experimental design limitations and/or interference from other constituents in the mixture. Moreover, current international guidelines (i.e., ICH S2 (R1), OECD) do not include the consideration of relative genotoxicity comparisons between test articles. Based on an evaluation of the state of the science at this time, CTP agrees that the standard battery of hazard identification assays cannot determine whether one mixture is more, or less, genotoxic than another. Regarding the use of ENDS aerosols, there is a significant degree of variability in experimental study designs used to directly assess ENDS aerosol exposures both in the literature and submitted by applicants. Differences in ENDS aerosol generation, exposure conditions, and biological models often make it difficult to directly compare between products within a study and between different studies. There are also major concerns with ENDS aerosol dosimetry and questions regarding the most effective ways to quantify exposures. For example, if mutagenic HPHCs are present in an ENDS aerosol, then those aerosols clearly present a mutagenic hazard. However, a reported negative result in an in vivo or in vitro study may be attributable to an ENDS aerosol exposure that is too low to reflect the known and potential hazards of the test article. The concentrations of toxicants, including genotoxicants, in aerosols may be outside the response range of the assay – but not the carcinogenic risk range to humans – and produce a false negative result because the assay is just not sensitive enough. Dosimetry concerns and insufficient assay sensitivity may render results of genotoxicity assays inconclusive and unable to inform potential genotoxicity hazards of the tested ENDS.

The cytotoxicity of solvents or other ENDS constituents in e-liquid or aerosol may also confound results in genotoxicity assays. Cytotoxic constituents may limit the maximum concentration of test article an applicant can use in the assay. This creates an inadequate dosing regimen where a significant number of cells in the assay die before a genotoxic concentration is reached. When using a cytotoxicity-limited concentration of test article, the genotoxicity of known or unknown genotoxic constituents in the test article may not be discernable and the resulting data inaccurately supports a negative or equivocal call. For example, the inherent toxicity of some constituents present in a complex mixture has been shown to decrease Ames assay sensitivity and the ability to detect low-level genotoxicants (Kenyon et al., 2007; Misra et al., 2014). Also, nicotine is cytotoxic at concentrations commonly found in ENDS (Misra et al., 2014). Nicotine, or other cytotoxic constituents, present in the test article (i.e., e-liquid, aerosol, or aerosol condensate) may therefore interfere with the ability to detect a “true positive” genotoxic response. Genotoxicity assays for hazard identification generally require a full dose response range including high levels of exposure to constituents of concern. Therefore, it is plausible that cytotoxic constituents, such as nicotine, may be dose limiting for constituents of concern when a complex mixture (i.e., ENDS e-liquid, aerosol, or aerosol condensate) is evaluated as the test article. The levels of a

particular constituent of concern may be too low to accurately test whether the component is genotoxic or not. This will impede the ability of the assay to identify all hazards that are present and limits the overall utility of the genotoxicity assay. This dosing limitation is especially relevant to the toxicological evaluation of tobacco products because of the expected duration of use by consumers. Since nicotine is addictive, tobacco product cessation is often unsuccessful, and consumers commonly use tobacco products for decades. Since the total exposure to a toxicant affects the incidence or severity of adverse effects (i.e., Haber's Law) (Gaylor, 2000), levels of genotoxicants that are too low to produce positive results in a genotoxicity assay for hazard identification may still produce genotoxic effects in humans if the exposure time is prolonged. As genotoxic constituents act through a variety of mechanisms (e.g., oxidative stress, DNA damage) and have differing potencies for genotoxic effects, situations may arise where a genotoxic constituent may appear to have a no-effect threshold, where no genotoxic effect is observed when the constituent is delivered in sufficiently low amounts. However, to be confident that the data indicates a true no-effect threshold, the dosing regimen and cytotoxicity concerns outlined above must be addressed.

Genotoxicity assays for hazard identification performed using ENDS e-liquids, aerosols, or aerosol condensates as a test article may provide scientifically valid results (i.e., reliable genotoxic vs. nongenotoxic hazard identifications) if the concerns above are addressed and the study is demonstrated to be reliable. The ENDS PMTA guidance recommends using ICH S2(R1) as a guide for genotoxicity assessment of ENDS and suggests including comparator products in in vitro assays. However, the utility of these results in a subsequent cancer risk assessment is limited. As previously mentioned, genotoxicity assay results are used for hazard identification and results cannot be used to make relative comparisons between different tobacco products outside of qualitative determinations (e.g., both positive, both negative, one positive and one negative). For example, positive genotoxicity assay results using an ENDS aerosol condensate and a combusted cigarette smoke condensate are considered equivalent. The testing of e-liquid or aerosol mixtures using a genotoxicity assay developed for hazard identification purposes does not identify specific hazards present within the test article and does not inform or contribute to subsequent cancer risk assessments. Furthermore, as previously discussed, the presence of known genotoxic aldehydes in the aerosol is sufficient to conclude the whole mixture as genotoxic. As such, the use of ENDS e-liquid, aerosol, or aerosol condensate as a test article in these hazard identification assays provides little useful information for hazard identification of specific constituents, such as ingredients and leachables, especially considering the technical concerns outlined above. If an applicant submits genotoxicity data for hazard identification using whole ENDS e-liquids, aerosols, or aerosol condensates as a test article, reviewers should assess whether the applicant provided sufficient supporting data, justification, and scientific rationale to address the toxicological concerns noted in this memorandum. If within your review you determine that the applicant did not provide such supporting data with studies using whole ENDS e-liquids, aerosol, or aerosol condensates, toxicology reviewers should provide applicants with a deficiency in Cycle 1 of review requesting this supporting data.

ENDS e-liquids are complex mixtures that contain numerous constituents. Generally, chemical mixtures can be classified as being either an intentional mixture, a mixture originating from a single source, or a mixture originating from multiple sources and through multiple pathways (EC, 2012; Kienzler et al., 2017). Intentional mixtures have a well-known, defined composition. Mixtures originating from a single source and mixtures originating from multiple sources and through multiple pathways are unintentional

mixtures that have a variable composition and contain numerous unknown or unidentified substances (Kienzler et al., 2017). Unintentional mixtures are generally environmental samples, such as drinking water, wastewater, soil, and ambient air. Taking into consideration the concerns noted above and the currently available scientific information regarding the evaluation and assessment of chemical mixtures, OS considers ENDS e-liquids to be intentional mixtures formulated by applicants that have a known and defined composition.

Regulatory assessments of intentional mixtures "are based on the properties of the constituents supplemented, where appropriate, by tests carried out on the entire product" (EC, 2012). Notably, due to the considerations discussed above, tests using the entire product (e-liquids, e-liquid aerosols) may be of limited relevance to the genotoxicity evaluation of ENDS. Examples of intentional mixtures having a known composition include personal care products, food additives, and pesticides (Kienzler et al., 2017). This methodology of assessing an intentional mixture based on the individual constituents in the mixture is supported in several regulatory and legislative documents published by the European Union (Bopp et al., 2018; EC, 2012; Kienzler et al., 2017; Kienzler et al., 2016). DNCS also considers the guidelines for assessment of chemical mixtures outlined by the OECD (OECD, 2018) and the European Commission, as well as the guidelines outlined by EFSA and the World Health Organization (WHO) for testing the genotoxicity of mixtures (WHO, 2009) appropriate for the evaluation of ENDS intentional mixtures. The guidelines from EFSA and WHO were developed to address genotoxicants present in mixtures. Typically, genotoxicity hazards in known mixtures are identified by evaluating each individual constituent (EFSA et al., 2019). The EFSA guidelines primarily addresses specific issues related to genotoxicity hazard identification of mixtures and provides a general framework for these assessments. WHO recommends applying the tiered approach to mutagenicity testing described by EFSA (WHO, 2009). WHO also recommends that genotoxicity hazard identification for well-characterized mixtures (i.e., mixtures having constituents that are identified and quantified) use a component-based approach that separately evaluates all components individually (WHO, 2009). This component-based evaluation may be supplemented with quantitative structure-activity relationship (Q)SAR models for hazard identification purposes, and, where appropriate, a quantitative approach assuming risk addition of identified hazards will be used as a default for risk characterization (WHO, 2009). Risk assessment using risk or response addition may be used to assess the cumulative risk of toxicity of a mixture made up of constituents having similar adverse health outcomes (e.g., cancer). Risk or response addition is a process in which the individual mixture constituents are scaled by their relative potencies and then added together to estimate an overall cumulative ELCR (Beronius et al., 2020; USEPA, 2000). Although this approach assumes a lack of synergy, in which the combination of individual risks is more than additive, or antagonism, in which the combination of individual risks is less than additive, it allows the determination of a total, cumulative effect produced by a known mixture and will provide informative data to evaluate the overall risk of carcinogenicity posed by the new product.

Assessment and evaluation of mixtures is generally categorized into two classes, either using a whole mixture approach or a component-based approach (Kumari et al., 2020). A whole mixture is a mixture that is evaluated in its entirety and typically with exposure levels for the entire mixture unadjusted for any differences in toxic potencies of the mixture's component chemicals (USEPA, 2007). Whole mixtures may be defined and reproducible (e.g., where the process that created them is well understood), or the whole mixture may be defined by the presence of groups of structurally similar chemicals that often co-

occur (e.g., total chromium, total petroleum hydrocarbons) (USEPA, 2007). The term "whole mixture" is commonly applied to "highly complex mixtures with components that cannot be fully identified or reproducibly measured," such as diesel exhaust and gasoline. Whole mixture assessment methods (e.g., mixture Reference Doses [RfDs], Reference Concentrations [RfCs], and cancer slope factors) treat the whole mixture as a single entity and are similar to the way single chemicals are assessed (USEPA, 2007). Because of this, dose-response information is needed for the whole mixture. This whole mixture approach is generally used for evaluating complex mixtures with an incompletely defined composition (e.g., diesel exhaust, gasoline) and is best applied to mixtures having a constant composition over the entire exposure period. Toxicity information generated from whole mixture methods may reflect the toxic effects of unidentified or unknown chemicals that are present in the mixture, as well as the occurrence of chemical interactions and joint toxic action (e.g., synergism, additivity, antagonism) among chemicals in the mixture. However, the observed toxic effect (e.g., genotoxicity) of the whole mixture may be reduced due to interactions within the sample media and the occurrence of other toxicities (e.g., cytotoxicity) caused by specific constituents present in the mixture. The occurrence of these additional toxic responses may mask the observed overall toxicity of the mixture and limit the responsiveness and sensitivity of the specific assay being used (e.g., a mixture containing known genotoxicants is reported as non-genotoxic) (Escher et al., 2020; Judson et al., 2016; USEPA, 2007). With this approach, whole mixture effects can be evaluated by testing the mixture itself, or by using data produced from a mixture having a similar composition (Bopp et al., 2018). In a component-based approach, constituents within the mixture are viewed as a group of distinct components (e.g., single chemicals) and the effects of chemical groups are based on the individual components (Kumari et al., 2020). A component-based assessment uses single chemical exposure and dose response information to assess the toxicological properties of the overall mixture (USEPA, 2007). Previous studies using whole mixtures have focused on environmental, dietary, or consumer products; however, a component-based approach is generally used when the components of the mixture are known (Bopp et al., 2018).

Generally speaking, the relevant documents describing the toxicological evaluation of mixtures recommend applying a component-based approach for regulated products that contain fully defined or characterized mixtures. As specifically stated by EFSA regarding the genotoxicity assessment of chemically fully defined mixtures, "[f]or chemically fully defined mixtures, the Scientific Committee recommends applying a component-based approach, i.e., assessing all components individually using all available information including read across and quantitative structure-activity relationship [QSAR] considerations about their genotoxic potential, following the Scientific Committee guidance. This means that for regulated products, conclusions on genotoxicity will be required for all components or at least for representative chemical substances for mixtures containing structurally related chemicals"(EFSA et al., 2019). It is important to note that this does not mean all components or constituents of these mixtures are required to be known, and for products with mixtures that have uncharacterized components, those fractions may be tested separately from the complete mixture. In such a component-based approach, the constituents, ingredients, or chemicals contained in the mixture are assessed individually for their potential genotoxicity (EFSA, 2011; EFSA et al., 2019). Furthermore, a component-based approach for assessment of mixtures containing genotoxic constituents is in alignment with a generally accepted approach for assessment and evaluation of defined, intentional mixtures (Bopp et al., 2018; Kienzler et al., 2017; Kienzler et al., 2016).

Genotoxicity assessments using ENDS e-liquids, aerosols, or aerosol condensates as test articles limit the ability to confidently identify hazards and perform a thorough genotoxicity evaluation. Taking into consideration the concerns outlined above and the relevant scientific consensus, DNCS recommends that ENDS, more specifically e-liquids, be treated as intentional mixtures. Therefore, genotoxicity hazard identification will involve two assessments: one for e-liquid constituents (e.g., ingredients and leachables) and another for constituents identified in the ENDS aerosol. In alignment with the scientific consensus regarding regulation of products consisting of intentional mixtures, a component-based approach is used where all e-liquid constituents (i.e., ingredients and leachables) are individually assessed for genotoxicity hazards. As previously noted, e-liquid aerosolization generates several known genotoxic HPHCs. HPHC and other constituent yields are quantitatively reported as part of the PMTA submission. As such, an exposure assessment and estimated cancer risk evaluation for each genotoxic e-liquid and aerosol constituent can be performed separately, and then all the data will be incorporated into the cancer risk evaluation. This approach allows toxicology reviewers to identify genotoxic constituents in ENDS and evaluate the contribution of these constituents to the overall cancer risk of ENDS in PMTA review.

Role of Computational Toxicology for Genotoxic Hazard Identification

When implementing an integrated WOE approach, it is anticipated that there will be cases where empirical data may be either limited or inadequate to identify genotoxicity hazards to support regulatory toxicology assessments of ENDS constituents. In such instances, computational toxicology methods, such as [Q]SAR, can provide component-based analysis (including component-based analysis of mixtures) on the potential for genotoxicity to support regulatory toxicology decisions (WHO, 2009). It is essential that toxicology reviewers assess any applicant-provided accounting of human expert judgement (i.e., human interpretation of toxicity predictions) as well as consider and provide their own accounting of human expert judgement used in this evaluation, as it is a key factor in assessing conclusions of biological relevance for any computational prediction outcome (Honma et al., 2019; ICH M7(R2) 2023). An overview of the role and applied regulatory use of computational toxicology analysis in the context of tobacco product constituents will be provided in a future DNCS memorandum. The goal of this memorandum will be to provide toxicology reviewers with a foundation of computational toxicology that is aimed at describing the utility and interpretation of chemical structure activity relationships in assessing the toxicity hazards for specific chemicals. It will also discuss factors and criteria that can inform the interpretation of computational model outputs submitted by applicants as a component of regulatory product review.

Tiered Weight of Evidence Approach for Carcinogenicity Evaluations

CTP is implementing a WOE approach for hazard identification. As previously noted, our experience from review of PMTAs indicate that other constituents in ENDS (e.g., e-liquid ingredients, leachables from the container or packaging components), along with those on the established HPHC list, have the potential to confer toxicological risk for adverse health effects, including cancer risk. A WOE approach uses the totality of carcinogenicity and genotoxicity data, including but not limited to any data available in the literature or provided by the applicant, for a given constituent to determine the overall confidence in a hazard identification. A tiered WOE approach for hazard identification should be

employed, as discussed below, to evaluate the strength of evidence for carcinogenic hazards associated with ENDS constituents.

When determining the WOE, reviewers need to evaluate genotoxicity study designs and their statistical analysis for all publicly available and applicant-submitted studies. This includes, but is not limited to, number of replicates included in an in vitro assay or number of animals/sex/groups in an in vivo assay, power and sample size calculations, the use of appropriate controls and solvent vehicles, whether the controls used are within the historical control database for the specific laboratory, and the overall reproducibility and replicability of the study. Additionally, toxicology reviewers need to consider if applicant-submitted studies deviate from current guidelines that represent the scientific consensus on relevant issues (e.g., OECD, ICH S2 R1) and how any deviations affect the weight of the study. A scientific rationale is necessary if a study with insufficient statistical power or with an improper or inferior study design is included in an overall WOE analysis. This evaluation assesses the quality of the data that will be included in the WOE and identifies any relevant caveats or limitations associated with the data.

When evaluating data to assess WOE, for consistency among reviews, toxicology reviewers should consider the following points:

- Unless there are strong scientific reasons that raise concerns about the interpretability of study results, generally, in vivo studies carry more weight than in vitro studies and in vitro studies carry more weight than computational predictions.
- One or more relevant, properly conducted genotoxicity studies outweigh studies that are equivocal or not properly performed. In particular, the reviewer needs to consider the WOE, focusing on the overall relevance and quality of each genotoxicity study for an ENDS constituent.
- If there are two well-performed studies with clear and contradictory results, one positive and one negative, toxicology reviewers need to consider the whole data set to be equivocal. Additionally, as explained in OECD test guidelines, results from standard genotoxicity assays are typically represented as positive, negative, or equivocal. Toxicology reviewers should consider the relevant OECD test guidelines and discuss study findings in terms of OECD test guideline outcomes in their discipline reviews. Notably, in general, equivocal findings or data sets raise concerns, unless an applicant provides sufficient evidence and justification to distinguish between positive and negative responses.
- Prior genotoxicity hazard evaluations by regulatory agencies may be re-considered when taking into account new information. Existing hazard evaluations may be based on conflicting genotoxicity studies, limited data, and/or problematic study designs. New information provided by an applicant or found in the literature may impact a previous determination. It is also important to note that public health agencies often publish information on chemical exposures, particularly flavor chemicals, in the context of food additives (i.e., oral route of exposure), which may not be relevant for inhaled tobacco products. Notably, a finding of “Generally Recognized as Safe” (GRAS) by the United States FDA, private GRAS determinations, or “acceptable” by JEFCA, for food products intended to be consumed orally does not apply to the use or consumption of tobacco products, particularly inhaled products such as ENDS, or to the individual ingredients and constituents included in tobacco products.

- Several factors should be considered when comparing inhalation exposure to oral exposures. Inhaled chemicals are not subject to first-pass metabolism in the liver prior to entering the blood stream and systemic circulation and may be bioactivated locally in the lung or conducting airways. These factors can affect target tissue exposure. Additional information on route of administration and target tissue exposure concerns are detailed in Section 3.2 of OECD document on the conduct and design of chronic toxicity and carcinogenicity studies (OECD, 2014) and by the EFSA Scientific Committee (EFSA et al., 2017). It is necessary for toxicology reviewers to verify that a provided carcinogenicity study used a relevant route of exposure for the reported endpoint, and that in vivo studies of genotoxicity provide data confirming that the sampled tissue was exposed to the test article being evaluated.
- Reviewers need to consider the mechanisms that different kinds of genotoxicity assays interrogate. For example, an Ames assay detects DNA mutations induced by DNA-reactive genotoxic substances (mutagens), while the rodent MN assay (in vitro or in vivo) measures a test article's potential for genotoxicity induced by clastogenic or aneugenic effects. If an Ames assay is positive, while a rodent MN assay is negative, the test article in question is still considered mutagenic because of the different mechanisms interrogated by these assays.

Reviewers should evaluate each constituent (e.g., HPHCs, ingredients, and leachables) individually and identify relevant genotoxicity data. Following a hazard identification assessment that incorporates genotoxicity data, constituents are tiered based on all available scientific data. As stated previously, scientific data used by reviewers for individual constituent toxicological evaluation and subsequent tiering should include, but is not limited to, data provided by applicants in product applications, studies found in the literature, and information contained within publicly available databases. Constituents lacking sufficient and adequate data for a toxicological evaluation should be assessed computationally and with additional studies submitted by the applicant. If applicants provide an alternate justification to indicate why they conclude that a constituent is not a genotoxic hazard this information should be evaluated as part of the toxicology review. Following the toxicological assessment of individual constituents contained in the new product, the overall carcinogenic risk expected with use of the new product will be evaluated using the tiering approach and through the calculation of the cumulative ELCR associated with the new product (See FDA memorandum: Calculating Excess Lifetime Cancer Risk in Tobacco Product Applications, June 3, 2024). This tiering approach takes into consideration our confidence in the accuracy of these data, as they pertain to the constituent's carcinogenic potential. Regarding carcinogenicity, the EPA and IARC both recommend a tiered approach that utilizes WOE analyses for their ultimate hazard identification methodology (IARC, 2019a; USEPA, 2005).

For tiering, EPA and IARC recognize three broad categories of data including 1) human data (e.g., primarily epidemiological studies), 2) long-term (i.e., chronic) experimental animal bioassays, and 3) supporting data (e.g., short-term [i.e., acute] tests for genotoxicity and other relevant properties, pharmacokinetic and metabolic studies, and structure-activity relationships). To classify ENDS constituents, CTP intends to use a tiering system that is consistent with those outlined by EPA and IARC, and modified as necessary to reflect CTP's regulatory scope. This modified tiering system is necessary for CTP toxicology reviewers to evaluate the potential carcinogenic hazards posed by ENDS constituents, some of which are commonly found to have limited toxicological data available and have not been evaluated for carcinogenicity by EPA or IARC. To that end, human data, animal data, and supporting

evidence are evaluated to characterize the WOE for carcinogenicity, and constituents are then placed into one of five tiers, namely:

Tier 1: Carcinogenic to humans

Tier 2: Likely to be carcinogenic to humans

Tier 3: Suggestive evidence of carcinogenic potential

Tier 4 (A-E): Potential carcinogenic hazard

Tier 5: Unlikely to contribute to carcinogenic risk of ENDS

Tiers 1-3 are limited to constituents previously classified by EPA and IARC, as described below. Constituents not evaluated by EPA or IARC but with data that support a potential carcinogenicity or genotoxicity concern, or constituents classified as EPA Group D or IARC Group 3, are classified as Tier 4. Tier 4 constituents are further categorized into subgroups A-E based on the strength of supporting data as detailed below. Constituents that are not likely to contribute to the carcinogenic risk of ENDS, based on review of the available evidence, fall into Tier 5. After all identified hazards are classified by tier, toxicology reviewers should perform an ELCR assessment as outlined in the related DNCS memorandum (see Memorandum: Calculating Excess Lifetime Cancer Risk in ENDS Premarket Tobacco Product Applications, June 3, 2024). Tiering considerations and guidelines are described below and include examples of the types of data found in each tier.

The tiering considerations described below incorporate carcinogenicity classifications made by EPA and IARC. There may be instances where the EPA and IARC classifications are in conflict (e.g., EPA classification indicates Group A, while IARC indicates a Group 2A classification). EPA and IARC classifications may be updated to reflect new toxicological information, and newly available data may alter previous classifications; as such, toxicology reviewers should look for and use the most recently published EPA or IARC classification (see Table 1 and Table 2, respectively). A pertinent example for ENDS is acrolein, which IARC had previously categorized as ‘not classifiable as to its carcinogenicity in humans (IARC Group 3)’ (IARC, 1995) but recently changed to ‘probably carcinogenic to humans (IARC Group 2A)’ (IARC, 2021) based upon in vivo evidence of carcinogenicity in experimental animals and strong mechanistic evidence primarily from in vitro studies.

Each tier is described in detail below. The WOE descriptors “sufficient,” “strong,” and “limited” are defined in Table 3.

Tier 1 WOE considerations: Carcinogenic to humans

Tier 1 classifications are limited to constituents classified as EPA Group A or IARC Group 1. Toxicology reviewers should not classify constituents that have not been previously evaluated by EPA or IARC into Tier 1. Constituents included in Tier 1 are carcinogenic to humans and have a WOE that demonstrates strong evidence of human carcinogenicity. This may include having convincing epidemiological evidence of a causal association between human exposure and cancer, or less weight of epidemiological evidence

that is supported by other evidence. Factors used by EPA and IARC for such classifications includes either:

- *Convincing* epidemiological evidence of a causal association between human exposure and cancer or
- A lesser weight of epidemiologic evidence that is strengthened by all the following lines of evidence:
    - *Strong* evidence of an association between human exposure, and either cancer or the key precursor events of the constituent's mode of action, but not enough for a causal association,
    - *Extensive* evidence of carcinogenicity in animals,
    - The mode(s) of carcinogenic action and associated key precursor events have been identified in animals, and
    - There is *strong* evidence that key precursor events that precede the cancer response in animals are anticipated to occur in humans and progress to tumors, based on available biological information.

Tier 2 WOE considerations: Likely to be carcinogenic to humans

Tier 2 classifications are limited to constituents classified as EPA Group B1/B2 or IARC Group 2A. Toxicology reviewers should not classify constituents that have not been previously evaluated by EPA or IARC into Tier 2. Constituents included in Tier 2 are likely to be carcinogenic to humans and have a WOE that demonstrates carcinogenic potential to humans but does not reach the WOE for the descriptor "Carcinogenic to Humans." As described by the EPA, the term "likely" acts as a WOE descriptor and represents a broad range of data combinations (USEPA, 2005). Factors used by EPA and IARC for such classifications include:

- *Plausible,* but not definitively causal, association between human exposure and cancer, with *some* supporting biological, experimental evidence, though not necessarily carcinogenicity data from animal experiments,
- Positive results in animal experiments in more than one species, sex, strain, site, or exposure route, with or without evidence of carcinogenicity in humans,
- Positive tumor study results that raise additional biological concerns beyond that of a statistically significant result (i.e., finding a high degree of malignancy or an early age of cancer onset),
- A rare animal tumor response in a single experiment that is assumed to be relevant to humans, or
- Positive tumor study that is strengthened by other lines of evidence such as:
    - Plausible association between exposure and cancer in humans, or
    - Known metabolite/s associated with tumor formation.

Tier 3 WOE considerations: Suggestive evidence of carcinogenic potential

Tier 3 classifications are limited to constituents classified as EPA Group C or IARC Group 2B. Toxicology reviewers should not classify constituents that have not been previously evaluated by EPA or IARC into Tier 3. Constituents included in Tier 3 have suggestive evidence of carcinogenicity to humans and have a WOE that demonstrates a concern for potential carcinogenic effects in humans, however the available data are judged to be not sufficient for a Tier 1 or Tier 2 classification. The WOE for Tier 3 constituents includes evidence associated with varying levels of concern for genotoxicity and carcinogenicity. Data that may support a Tier 3 classification includes in vivo data indicating a positive cancer result in the only study evaluating a specific constituent or a single positive cancer result in an extensive database that includes negative studies in other animal species. Strong in vivo evidence that a constituent exhibits key precursor events considered integral to the carcinogenic process is also sufficient for a Tier 3 classification. Factors used by EPA and IARC for such classifications include:

- *Limited* evidence of an association between human exposure and either cancer or the key precursor events of the constituent's mode of action,
- *Sufficient* evidence of carcinogenicity in animals,
- *Strong* evidence that the key precursor event(s) occur within experimental systems (e.g., in vivo that potentially precede the cancer response).

Tier 4 (A-E) WOE considerations: Potential carcinogenic hazard

Constituents in Tier 4 have demonstrated carcinogenicity or carcinogenic potential. Tier 4 constituents have not been formally evaluated by EPA or IARC to assess their carcinogenicity or, if they have previously been evaluated by EPA or IARC, may be classified as EPA Group D or IARC Group 3. Constituents classified as EPA Group D or IARC Group 3 could fall into any Tier 4 subgroup depending on the evidence used to make the EPA/IARC determination.

A broad range of evidence, including human, animal, in vitro, and computational data, is appropriate for a Tier 4 classification. Constituents with a positive result from a carcinogenicity study (e.g., human clinical data, in vivo study), an in vivo genotoxicity hazard identification assay (e.g., in vivo MN), an in vitro hazard identification assay (e.g., Ames, mouse lymphoma, MN, comet), or a computational assessment fall into Tier 4. A single positive result from a new approach methodology (NAM) may also be sufficient for a Tier 4 classification. NAMs include a broad range of "alternative methods" such as in vitro, in chemico (i.e., assays that identify reactive chemicals), in silico (e.g., computational, bioinformatics), and systems biology approaches. For example, positive genotoxicity hazard identification results produced using computational methodologies that predict the outcome of a bacterial mutagenicity assay (i.e., Ames assay) are placed in Tier 4. Computational approaches involving (Q)SAR methodologies should follow generally accepted validation principles such as those set forth by OECD. In vivo methods can also be considered NAMs when they improve predictivity, shift studies to phylogenetically lower animals, or help replace, reduce, and refine animal use. NAMs are currently being applied to the field of chemical risk assessment to generate more complete and comprehensive datasets regarding the safety of chemicals (Kavlock et al., 2018; Westmoreland et al., 2022). Utilizing in vitro assays, NAMs, and computational toxicology approaches is a critical component of the "Toxicology in

the 21st Century" (Tox21) effort to move towards using non-animal experimental methods and a key tool in the generation of experimental data for the vast array of data-limited constituents used in tobacco products.

A general barrier to the use of NAMs for assessment of exposure risks is a lack of broad acceptance of the new methodologies and a lack of validated protocols and procedures (Kavlock et al., 2018). NAMs have also been developed specifically for use in assessing the genotoxic potential of a chemical (Fortin et al., 2023). NAMs may provide useful scientific information in a WOE-based evaluation of an ENDS constituent's genotoxic potential. Toxicology reviewers should first determine whether the specific NAM being used has been validated or incorporated into existing guidelines (e.g., OECD or ICH). A positive result in a validated NAM with supporting expert judgement is sufficient to support a Tier 4 classification. Results from NAMs can also be used to support a Tier 5 classification, as described below. Importantly, results from a carefully conducted NAM that has not been validated could be considered equivalent to a validated method for tiering purposes; however, the method and results must be evaluated on a case-by-case basis. In their assessments, the reviewer should take into consideration the specific genotoxicity mechanism and genotoxic outcome being evaluated in the NAM. This information should be considered in relation to other genotoxicity data submitted for the new product and whether these other assays use recognized and/or validated methodologies.

In sum, specific factors for the reviewer to consider are listed below. Note that this is not an exhaustive list and that NAMs should be evaluated on a case-by-case basis:

- Has the NAM been rigorously validated (ICCVAM, 2024)?
- Is an appropriate dose or exposure range used?
- Is the model system used appropriate for the outcome being measured?
- How does the NAM assay outcome relate to genotoxicity, and does it involve established genotoxic mechanisms or pathways?
- Is the reported result from the NAM consistent with other genotoxicity assays used to evaluate the specific constituent?
- If the NAM is an in vitro or in vivo assay, is the NAM result supported by computational toxicology assessment?

When classifying Tier 4 constituents, reviewers will carefully consider the available evidence, including apparent conflicting evidence, in the context of the WOE considerations discussed above. Notably, equivocal findings or data sets raise concerns unless there is sufficient evidence and justification to distinguish between positive and negative responses. When classifying constituents into Tier 4, reviewers will also consider what types of data would be helpful for reclassification to Tier 5. For Tier 4 constituents, it is possible that follow-on assays beyond the currently available data may resolve identified genotoxicity or carcinogenicity concerns. Detailed decision trees, in alignment with the WOE considerations discussed above, that describe when follow-on assays may outweigh identified concerns for carcinogenicity or genotoxicity are included in the Appendix.

Tier 4 is divided into five subgroups (A-E) based on the certainty of the supporting data to reflect varying levels of concern for carcinogenicity. Toxicology reviewers should classify such constituents under the

highest applicable Tier 4 subgroup but ensure that identified concerns from all Tier 4 subgroups are addressed in their review. For example, based upon the criteria described below, a constituent with a positive in vitro Ames and a positive in vitro MN assay would be classified as Tier 4B rather than Tier 4C. However, toxicology reviewers should address the concern for clastogenicity or aneugenicity from the positive in vitro MN assay along with the mutagenicity concern from the positive Ames assay in both the discipline review and, if applicable, any communication to the applicant.

Tier 4A:

Constituents in Tier 4A have evidence of carcinogenicity or genotoxicity in humans or in vivo model systems. Minimally, a positive finding from an in vivo genotoxicity study (e.g., in vivo MN assay) is sufficient for a Tier 4A classification. A positive carcinogenicity finding (e.g., increased tumor incidence) in at least one human or animal study would also place a constituent in Tier 4A. Notably, these factors alone may not be sufficient for a higher level (i.e., Tier 1-3) classification. Tier 4A constituents may have stronger evidence for carcinogenicity beyond these factors, such as epidemiological evidence of carcinogenicity in humans or extensive evidence of carcinogenicity in animals. It is possible that such constituents with stronger evidence may meet the considerations for Tiers 1-3 described above. However, if such constituents have not been previously evaluated by EPA or IARC, they would nonetheless fall into Tier 4A under this tiering system. Under the considerations outlined for Tier 4A herein, there are various levels of certainty for a constituent's carcinogenicity in humans based upon the available evidence. For example, in the absence of other supporting data, there is more certainty for a constituent's carcinogenicity resulting from positive findings in a carcinogenicity study conducted by the National Toxicology Program than positive findings for the same constituent in an in vivo MN assay. Similarly, in the absence of other supporting data, there is more certainty for the carcinogenicity of a constituent that has positive findings from multiple two-year rodent carcinogenicity studies in different species than positive findings from a single carcinogenicity study for the same constituent. Toxicology discipline reviews should indicate the supporting evidence used to classify constituents into all tiers, including Tier 4A.

Tier 4B:

Constituents in Tier 4B have a positive finding for mutagenicity from at least one in vitro Ames assay. In vitro Ames data is included in the genotoxicity hazard identification assessment as this is an accurate predictor of tumor outcomes in vivo. The Ames assay is reported to have a positive predictive value, which ranges depending on the number of chemicals used to obtain the values, for carcinogenicity of 76-87% (EFSA, 2011; Zeiger, 1998). Furthermore, the use of the Ames assay to assess the mutagenicity of a constituent is supported by the ICH S2(R1) guidance for genotoxicity testing and data interpretation of pharmaceuticals intended for human use. Alternatively, computational structural alerts from (Q)SAR methodologies that predict the outcome of an Ames mutagenicity assay using either expert rule- or statistical-based methodologies would be appropriate for a Tier 4B classification. The ICH M7(R2) guidance, which discusses the assessment and control of mutagenic impurities in pharmaceuticals, notes that a (Q)SAR assessment that focuses on bacterial mutagenicity (i.e., Ames) predictions may be used when in vitro data are not available.

Tier 4C:

Constituents in Tier 4C have a positive finding from at least one other (i.e., non-Ames) in vitro genotoxicity assay. The ICH S2(R1) guidance for genotoxicity testing and data interpretation of pharmaceuticals intended for human use provides support and a rationale for the use of a battery approach to assess the genotoxicity of a constituent. This guidance indicates that including in vitro mammalian genotoxicity assays in a battery approach together with the Ames assay for mutagenicity increases the sensitivity for detection of rodent carcinogens and broaden the spectrum of genetic events detected, although this approach ultimately decreases the specificity of carcinogenicity predictions. ICH S2(R1) concludes that using a battery approach "is still reasonable because no single test is capable of detecting all genotoxic mechanisms relevant in tumorigenesis." Many in vitro assays beyond the Ames assay evaluate mutagenicity or other mechanisms of genotoxicity and are regularly used for regulatory evaluations. Relevant examples of such assays include, but are not limited to, the in vitro chromosomal aberration assay, the in vitro MN assay, and the in vitro mouse lymphoma assay, which are identified as appropriate assays to investigate chromosomal damage in ICH S2(R1) (ICH, 2011). The positive predictivities of these in vitro assays in detecting rodent carcinogens are 67-76%, 76-80%, and 66-74%, respectively (EFSA, 2011). A single positive result from a carefully conducted in vitro NAM, as described above, is also sufficient for a Tier 4C classification.

Tier 4D:

Constituents with positive non-Ames computational toxicology predictions for carcinogenicity or genotoxicity are classified as Tier 4D. When empirical data are lacking, computational toxicology evaluations can predict genotoxic hazards for constituents to support regulatory assessments. Several computational prediction models that evaluate various carcinogenicity, genotoxicity, or mutagenicity outcomes are freely or commercially available. Computational prediction outcomes may be provided by applicants in premarket submissions or performed by DNCS staff. Importantly, all prediction outcomes should be evaluated by human expert judgement in the context of the overall weight of evidence to minimize false positive findings. Additional considerations regarding computational toxicology for genotoxicity hazard identification are described above and will be discussed in further detail in a future DNCS memorandum.

Tier 4E:

Tier 4E constituents lack sufficient data for a Tier 5 classification and do not fit into any other tiers outlined herein. Factors for a Tier 5 classification are discussed in detail below.

In summary, when considering if a constituent is Tier 4, reviewers should include:

Tier 4A

- Constituents that meet the considerations for Tiers 1-3 described herein but have not been formally evaluated by EPA or IARC, or

- Constituents with at least one study (e.g., human clinical data, in vivo rodent carcinogenicity studies studies) demonstrating a positive carcinogenicity result, or
- Constituents with a positive, as defined by the relevant OECD Test Guidelines, result from at least one in vivo genotoxicity study.

Tier 4B

- Constituents with a positive, as defined by the relevant OECD Test Guidelines, result from an in vitro Ames mutagenicity assay, or
- Constituents with structural alerts from (Q)SAR methodologies that predict the outcome of an Ames mutagenicity assay using either expert rule- or statistical-based methodologies. Computational toxicology data from the use of these two complementary, validated (Q)SAR methodologies that predict the outcome of an in vitro bacterial mutagenicity assay (i.e., Ames assay) may be used to assess the mutagenic hazard of a constituent, in lieu of conducting the in vitro Ames assay itself. This computational approach is supported by ICH M7 (R2).

Tier 4C

- Constituents with a positive result, as defined by relevant OECD Test Guidelines, from a conventional non-Ames in vitro genotoxicity assay, or
- Constituents with a positive result from an in vitro NAM evaluating genotoxicity or mutagenicity.

Tier 4D

- Constituents with a positive result from a computational NAM, or
- Constituents predicted positive for carcinogenicity or genotoxicity using read across from a known carcinogen or genotoxicant, or
- Constituents predicted positive using computational models for any other in vivo or in vitro carcinogenicity or genotoxicity assay.

Tier 4E

- Constituents that have insufficient data available for a Tier 5 classification that do not fit into other tiers.

Tier 5 WOE considerations: Unlikely to contribute to carcinogenic risk of ENDS

Constituents in Tier 5, based on evidence available to reviewers, are unlikely to contribute to the carcinogenic risk of ENDS. Tier 5 constituents have available data that are robust and which reviewers can use to determine that there is no basis for a genotoxic or carcinogenic hazard concern in the context of ENDS premarket application review. In this classification, the WOE includes in vivo, in vitro, and computational evidence that the constituent is not likely to have carcinogenic or genotoxic potential in humans. A reviewer can consider several lines of evidence as appropriate for determining a Tier 5 classification.

Constituents classified as EPA Group E would be placed in Tier 5. For constituents with carcinogenicity studies available, evidence from well-designed and well-conducted animal studies that demonstrates a *lack* of carcinogenic effect via the inhalation route, in the absence of other animal or human data suggesting a potential for carcinogenic effects, are appropriate for a Tier 5 classification. Animal studies that demonstrate a lack of carcinogenic effect via other exposure routes may still be appropriate for a Tier 5 classification if accompanied by appropriate scientific justification to indicate that similar results would be expected by the inhalation route. Alternatively, if applicable, there may be *convincing* and *extensive* experimental evidence showing that positive carcinogenic effects observed in animals are not relevant to humans or c*onvincing* evidence that carcinogenic effects are not likely to occur by a particular exposure route.

When carcinogenicity data are not available for a constituent, a Tier 5 classification may be appropriate based upon genetic toxicology evidence alone. In such instances, in line with ICH S2(R1), such genetic toxicology evidence would include all the following if the WOE does not identify conflicting, positive, or equivocal results elsewhere:

- One negative finding for mutagenicity from an in vitro, in vivo, or computational test, and
- One negative finding for chromosomal damage from an in vitro or in vivo test, and
- No positive or equivocal predicted outcomes from computational toxicology evaluations.

When evaluating negative responses in hazard identification assays, toxicology reviewers should consult applicable test protocols and guidelines (e.g., the definition of clearly negative results as defined in OECD test guidelines) to evaluate the validity of such outcomes. Importantly, all negative hazard identification results should also be supported using any available scientific data from literature that describes the known toxicological or pharmacological properties of the constituent.

The above criteria based upon genetic toxicology evidence alone do not necessitate a negative finding from an in vivo genotoxicity test. In vivo studies are not necessary for a Tier 5 classification in this context, and, in most cases, in vivo studies are not necessary to serve as confirmatory assays. This deviates from the standard genotoxicity test batteries such as ICH S2(R1) but is appropriate in the context of the tobacco product application review as tobacco products are evaluated for overall health risks instead of safety. Notably, other regulatory bodies such as EFSA and the UK Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment highlight a similar step-wise approach to genotoxicity evaluations that first focuses on in vitro testing and only proceeds to in vivo testing if warranted based upon the available data (COM, 2021; EFSA, 2011).

Although in vivo studies are not necessary for there to be adequate genetic toxicology evidence for a Tier 5 classification, in vivo studies may be useful to provide additional information on the potential genotoxicity of a constituent if in vitro assays do not capture the potential genotoxic effects of the constituent, or to potentially outweigh positive in vitro genotoxicity results. As noted in the ICH S2(R1) guidance (ICH, 2011), following a positive result for genotoxicity from an in vitro mammalian cell assay, clearly negative results from two well-conducted in vivo assays, and with demonstrated adequate exposure, is one way to demonstrate sufficient evidence for lack of genotoxic potential in vivo. This in vivo approach requires confirming exposure of the target tissue that is being evaluated by the specific

genotoxicity assay being performed (i.e., route of exposure modifications may be needed to obtain systemic distribution and target tissue exposure).

Negative prediction outcomes (i.e., a lack of structural alerts) from a computational model of the Ames assay may be used to support a Tier 5 classification for constituents. The toxicology reviewer should note that negative prediction outcomes reported from computational toxicology assessments suggest the absence of structural alerts or other structure-activity relationships relevant to the specific toxicological hazard being evaluated (e.g., mutagenicity, genotoxicity, carcinogenicity). Although the ICH M7(R2) guidance was not specifically designed for tobacco products, this document provides information on evaluating computational toxicology assessments in hazard identification (ICH, 2023). In the absence of mutagenicity (Ames) data, ICH M7 states that a quantitative structure-activity relationship (QSAR) and structure-activity relationship (SAR) computational toxicology assessment may be used as a substitute for Ames test data. ICH M7(R2) states that "[t]he absence of structural alerts from two complementary (Q)SAR methodologies (expert-rule based and statistical) is sufficient to conclude that the [constituent] is of no mutagenic concern..." ICH M7(R2) further states, "[i]f warranted, the outcome of any computer system-based analysis can be reviewed with the use of expert knowledge in order to provide additional supportive evidence on relevance of any positive, negative, conflicting, or inconclusive prediction and to provide a rationale to support the final conclusion." Negative prediction outcomes should be carefully interpreted to minimize the risk of false negatives (e.g., chemicals that are falsely determined to have non-carcinogenic potential). The determination of a negative prediction outcome may be considered following the examination of a constituent's potential bioactivation and elimination pathways and may support the determination of minimal or low toxicity based on currently available knowledge and information. A constituent supported by an expert knowledge assessment of a negative prediction outcome could be determined to be of low toxicity concern (Tier 5). Notably, if there is supporting data such as relevant literature to indicate the formation of a toxic-, or perhaps potentially carcinogenic-, metabolite, a constituent could be reclassified (i.e., Tier 4D).

When classifying Tier 5 constituents, reviewers will carefully consider the available evidence, including prediction outcomes from computational models beyond a computational Ames assessment, in the context of the WOE considerations discussed above. If positive, equivocal, or conflicting evidence is identified for the constituents under consideration, a Tier 5 classification would not be appropriate unless accompanied by an adequate scientific justification to demonstrate that such findings are not of toxicological concern. In that regard, detailed decision trees in alignment with the WOE considerations discussed above are included in the Appendix that describe when negative results from follow-on assays may outweigh identified concerns for carcinogenicity or genotoxicity, and therefore these assay results could support a Tier 5 classification. The WOE for Tier 5 classifications also includes situations where positive results in experimental animals are determined to lack biological relevance with supporting scientific justification, such as when there is strong, consistent evidence that each mode of action in the experimental model does not operate in humans (e.g., sole mutations of genes that are not present in humans).

Tiering of constituents in toxicology reviews

Toxicology reviewers should use the above approach and considerations as well as their expert judgement to tier all constituents identified in ENDS PMTAs. ENDS PMTAs Toxicology discipline reviews should specify which tier a constituent is classified under and clearly indicate the supporting evidence used to make that determination. After all constituents identified as carcinogenic or genotoxic hazards are classified by tier, toxicology reviewers will perform an ELCR assessment as outlined in the related DNCS memorandum (see Memorandum: Calculating Excess Lifetime Cancer Risk in ENDS Premarket Tobacco Product Applications, June 3, 2024). A discussion of which genotoxic or carcinogenic hazards will move forward for the ELCR analysis is provided in detail in the decision trees in the Appendix. In general, Tier 4 constituents may raise concerns for carcinogenicity or genotoxicity. However, it is possible that additional evidence beyond the information that was originally available, such as additional evidence provided by an applicant in response to CTP communications, may allay carcinogenicity concerns for Tier 4 constituents and support a Tier 5 classification. Decision trees in alignment with the WOE considerations discussed above that describe when follow-on assays may outweigh identified concerns for genotoxicity or carcinogenicity of Tier 4 constituents and determine whether a specific constituent should be included within the cumulative ELCR assessment are provided in the Appendix. Some specific examples are as follows:

- For a Tier 4B constituent having positive (Q)SAR predictions for mutagenicity in a computational Ames assessment, an in vitro Ames assay may provide additional information to outweigh the computational assessment. In this scenario, negative results from an in vitro Ames assay may support reclassifying the constituent as Tier 5 if other adequate evidence for a Tier 5 classification is available, whereas positive results would support the carcinogenicity of the constituent.
- For a Tier 4D constituent having a positive prediction in a computational MN assay model, an in vitro MN assay may provide additional information to outweigh the positive computational findings. In this scenario, negative results from an in vitro MN assay may support reclassifying the constituent as Tier 5 if other adequate evidence for a Tier 5 classification is available, whereas positive results would support the carcinogenicity of the constituent.
- For a Tier 4E constituent with a negative in vitro MN assay but no available data regarding the mutagenicity of the constituent, negative prediction outcomes from a computational Ames assessment using both expert-rule based and a statistical-based QSAR methodology may support reclassifying the constituent as Tier 5 in the absence of other positive or equivocal data elsewhere (e.g., in vivo, in vitro, or computational data).

If additional information is provided beyond what was available in the original application (e.g., in a subsequent amendment submitted by an applicant), toxicology reviewers should evaluate this information to determine whether constituents can be reclassified into a different tier. Subsequently, toxicology reviewers should calculate an updated ELCR based upon the new constituent tiers and hazards as outlined in a separate DNCS memorandum (see Memorandum: Calculating Excess Lifetime Cancer Risk in ENDS Premarket Tobacco Product Applications, June 3, 2024), using the framework outlined in the decision trees in the Appendix to determine which hazards should be included in the cumulative ELCR assessment.

In summary, ENDS contain numerous constituents, some of which are genotoxic and/or carcinogenic. The purpose of the hazard identification and carcinogenicity tiering process outlined above is to identify

those tobacco product constituents that are genotoxic and/or carcinogenic and evaluate the carcinogenic risk these constituents pose to users of the new tobacco product. Following the identification of genotoxicity hazards and tiering of carcinogenic potential, the next step in the toxicological evaluation is to perform a cancer risk assessment. Specific information regarding the process and methodology for conducting this evaluation is described elsewhere in a DNCS memorandum (see Memorandum: Calculating Excess Lifetime Cancer Risk in ENDS Premarket Tobacco Product Applications, June 3, 2024). The approach outlined within this memorandum for genotoxicity hazard identification and constituent carcinogenicity tiering are crucial in estimating the cancer risk posed by ENDS in PMTAs.

Limitations

Hazard identification, risk characterization, and risk assessment are inherently dependent on the quality of the scientific data. The process described in this memorandum represents an informative, comprehensive, and science-based approach for assessing the genotoxicity and carcinogenicity of ENDS and aligns with the regulatory scope of CTP. However, there are limitations to this approach that the toxicology reviewers should take into consideration when evaluating ENDS PMTAs:

- Hazard identification and carcinogenicity tiering using the described component-based approach may not capture unknown pyrolysis products formed during aerosolization. Although a concern, temperatures in ENDS are much lower than combusted cigarettes and constituents, in particular flavoring compounds, tend to transfer efficiently and unchanged into the ENDS aerosol (e.g., mean transfer efficiency ≈ 98%) (Behar et al., 2018). Therefore, we expect there to be less pyrolysis of ENDS ingredients when compared to combusted tobacco products.
- Complete constituent information (e.g., single ingredients that comprise complex ingredients, identification of leachable compounds) may be lacking in PMTAs.
- Genotoxicity test batteries used for hazard identification are designed to detect potential carcinogens that primarily act through mechanisms involving DNA damage (ICH, 2011). As such, genotoxicity assays and test batteries are not expected to identify nongenotoxic carcinogens.

Conclusions

This memorandum outlines CTP's approach for reviewer evaluation and assessment of genotoxic hazards associated with the use of ENDS and addresses specific concerns identified during the toxicology review of new ENDS submitted through the PMTA pathway. Several toxicological concerns are discussed regarding the genotoxicity of ENDS, the hazard identification process, and the WOE tiering of potentially carcinogenic constituents. Toxicology reviewers should use the information provided in this memorandum and the resulting evaluations as part of their workflow to evaluate and compare the cumulative cancer risk posed by tobacco product constituents, as described in an accompanying DNCS memorandum (See Memorandum: Calculating Excess Lifetime Cancer Risk in ENDS Premarket Tobacco Product Applications, June 3, 2024). Although ENDS are the primary focus, the current memorandum may also be relevant to other types of new tobacco products submitted through the PMTA pathway (e.g., combusted tobacco products, smokeless tobacco products, oral nicotine products, non-tobacco nicotine products) and could potentially be used in reviews for other tobacco product categories that

cite a rationale for why the content in this memorandum is relevant to the evaluation of products under review.

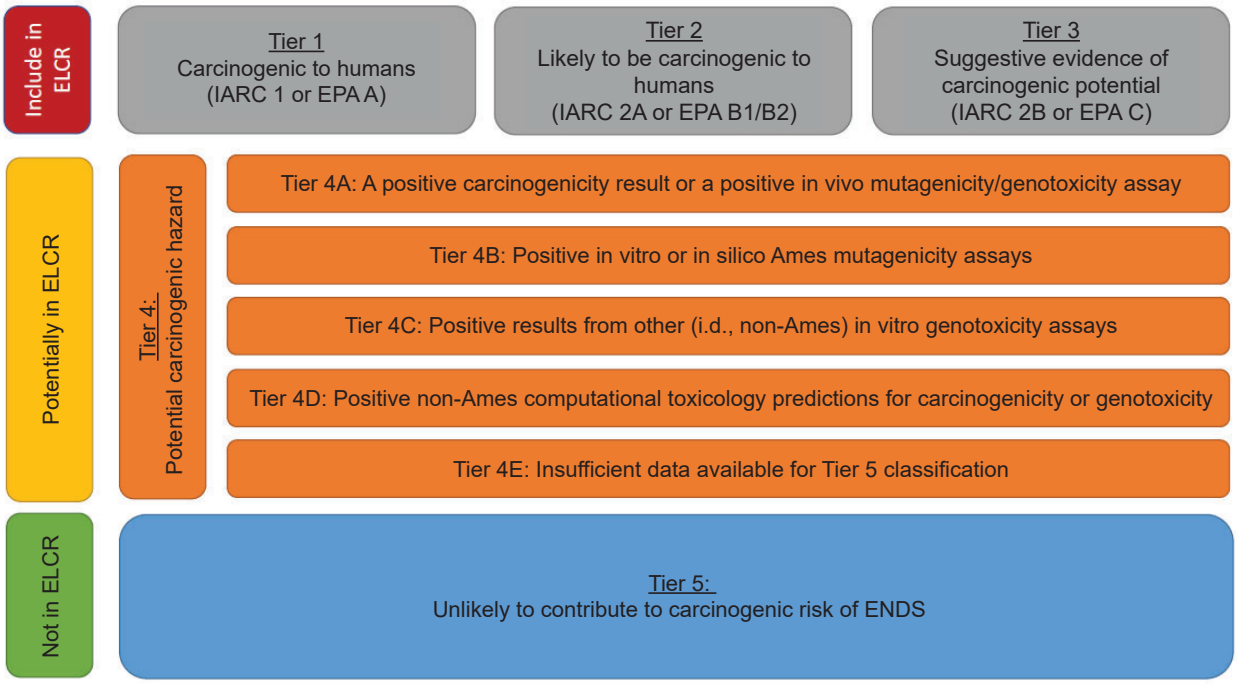
Moving forward, reviewers performing genotoxicity hazard identification and carcinogenicity assessments during their review of PMTAs should follow the framework outlined in this memorandum. DNCS intends to update this memorandum as needed to incorporate ongoing scientific developments in the assessment and evaluation of genotoxic chemicals and to ensure alignment with CTP and FDA mission requirements and priorities. Toxicology reviewers should perform genotoxicity hazard assessments from the standpoint that ENDS are complex known mixtures and, therefore, should be evaluated using a component-based approach. Genotoxicity hazard identification will precede categorizing the tobacco product constituents into tiers (Tiers 1 – 5) that are associated with the constituent's expected carcinogenic risk. The tiering system described within this memorandum is based on a conservative WOE approach and stratified by the overall confidence that a given constituent is likely to be carcinogenic. Hazard identification and tiering results for constituents (e.g., ingredients and leachables) are intended to be incorporated into a subsequent ELCR analysis that includes applicant reported HPHC yields to enable a holistic reviewer evaluation of the potential carcinogenic risk posed by the new product and facilitate comparative cancer risk evaluations.

Appendix

Acronyms

(Q)SAR	(Quantitative) Structure-Activity Relationship
APPH	Appropriate for the Protection of Public Health
ATSDR	Agency for Toxic Substances and Disease Registry
CCS	Container Closure System
CTP	Center for Tobacco Products
DNCS	Division of Nonclinical Science
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
ELCR	Excess Lifetime Cancer Risk
ENDS	Electronic Nicotine Delivery System
EPA	Environmental Protection Agency
FAO	Food and Agriculture Organization
FD&C	Food, Drug, and Cosmetic (FD&C) Act
FDA	Food and Drug Administration
GRAS	Generally Recognized as Safe
HPHC	Harmful and Potentially Harmful Constituents
IARC	International Agency for Research on Cancer
ICH	International Council for Harmonization
JEFCA	Joint FAO/WHO Expert Committee on Food Additives
NAMs	New Approach Methodologies
NCTP	Nonclinical Computational Toxicology Program
NIOSH	National Institute for Occupational Safety and Health
NTP	National Toxicology Program
OECD	Organization for Economic Cooperation and Development
PMTA	Premarket Tobacco Product Application
RfC	Reference Concentration
RfD	Reference Dose
SAR	Structure-Activity Relationship
WHO	World Health Organization
WOE	Weight of evidence

Decision trees for inclusion of a constituent in ELCR assessment following tiering classification



As described in the PMTA final rule,[3] under 21 CFR § 1114.7(k)(1)(i)(B), a PMTA must contain information describing "*the toxicological profile of the new tobacco product…including studies which discuss the toxicological effects of any leachables[.]*" The toxicological profile also includes information on the toxicity of the ingredients, additives, and HPHCs, relative to the route of administration and the range of potential levels of exposure resulting from the use of, or exposure to, the new tobacco product. The applicant must include toxicological information that is known, or reasonably expected to be known, in their PMTA. This information should include data and thorough literature reviews that address several health effects known to be caused by tobacco products. This includes, but is not limited to, information on the genotoxicity and carcinogenicity of the new product. Toxicology reviewers should review available information regarding the genotoxic and carcinogenic hazards of ENDS constituents and place them into Tiers 1-5 as outlined in this memorandum.

When evaluating carcinogenicity and genotoxicity data, whether applicant provided or publicly available, reviewers should consider the validity of all information by evaluating the study endpoints, protocols, and results as described in the WOE section above. As discussed earlier, a WOE approach that considers the totality of carcinogenicity and genotoxicity data for a given constituent is intended to be used. In the WOE approach, it is possible that stronger evidence may outweigh other available evidence, based upon the factors discussed above. Detailed decision trees for each tier that describe when certain information may outweigh identified hazards are described below. These decision trees may be used by toxicology reviewers when evaluating the totality of available evidence provided in PMTA submissions or, if applicable, subsequent amendments.

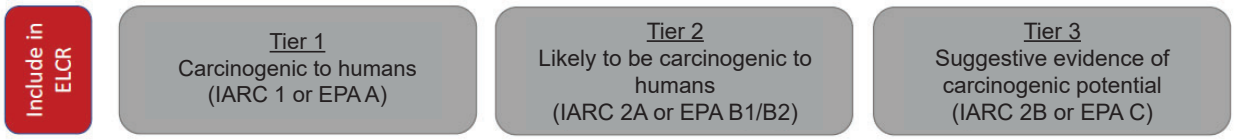
[3]Premarket Tobacco Product Applications and Recordkeeping Requirements October 2021. 86 Fed. Reg. at 55300 - https://www.federalregister.gov/documents/2021/10/05/2021-21011/premarket-tobacco-product-applications-and-recordkeeping-requirements

"Follow-Up" steps and communications to applicants

For these decision trees, "follow-up" steps have been included to provide reviewers information on what studies might outweigh identified concerns and allow reclassification of a Tier 4 constituent to Tier 5 if other Tier 5 criteria are met. These "follow-up" studies might already be available in the literature or in the submission under review. Additionally, in certain scenarios, computational analysis performed by the DNCS nonclinical computational toxicology program (NCTP) team may support "follow-up" steps for hazard identification. However, if these follow-up studies are not available, toxicology reviewers should work with the technical project lead (TPL) for the PMTA during Cycle 1 of review to provide communication to the applicant, if warranted, specifying the need for these follow-up studies to reclassify Tier 4 constituents to Tier 5. Notably, communication to the applicant regarding follow-up studies may not always be warranted. In Cycle 1 of PMTA review for a new product that has complete constituent information, it is possible to calculate a preliminary cumulative ELCR by including the constituents assigned to Tier 4 to assume a "worst case scenario." If the preliminary calculation places a new product in the "lower concern" category and below the median of the ENDS Marketing Granted Orders (MGO) marketplace as described in a separate DNCS memorandum (see Memorandum: Calculating Excess Lifetime Cancer Risk in ENDS Premarket Tobacco Product Applications, June 3, 2024), additional information from the applicant is unnecessary. In this scenario, any additional information provided by the applicant, such as data to reclassify Tier 4 constituents into Tier 5, is unlikely to change the conclusions of the cancer risk evaluation.

In cases where submitted studies indicate equivocal genotoxic or carcinogenic responses associated with a constituent, toxicology reviewers should provide information regarding the equivocal response to the TPL for communication to the applicant in Cycle 1. Toxicology reviewers should follow the appropriate decision trees for the indicated Tier 4 subgroup. If the applicant-provided information in response to CTP communications does not allay concerns, the reviewer should take into account all other sources of available information, including computational toxicology assessments, in a WOE approach. If the WOE supports that the constituent in question should be included in an ELCR finding – for example, if computational analyses indicate a potential for mutagenicity, clastogenicity, or carcinogenicity – the toxicology reviewer should calculate the ELCR with the constituent in question – and provide the TPL with a description of the WOE analysis that was conducted.

i. Tier 1-3 Constituents

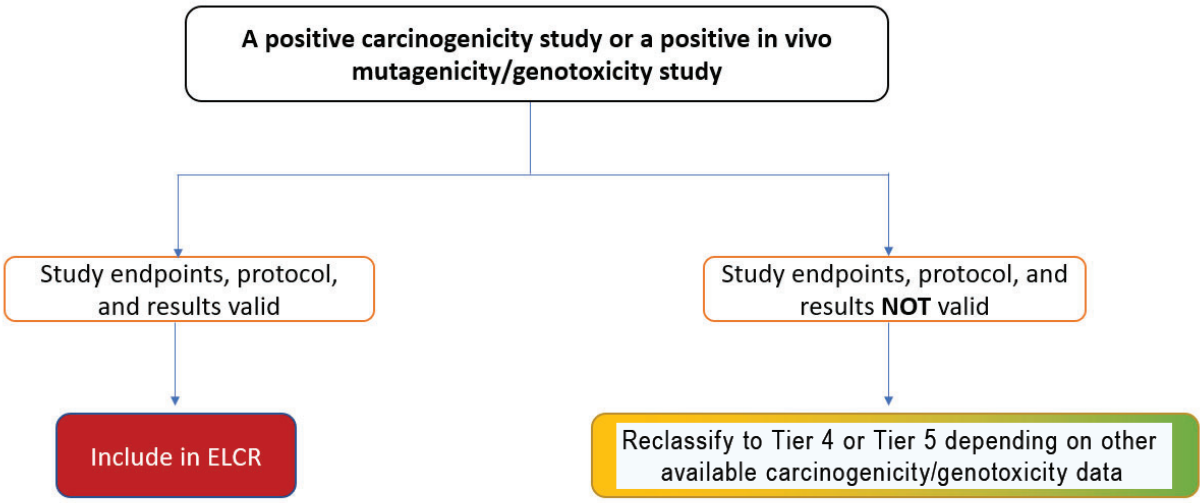


*Tier 1-3 constituents should be included in the ELCR unless, on a case-by-case basis, in alignment with the weight of evidence approach discussed above in "Tiered Weight of Evidence Approach for Carcinogenicity Evaluations," the toxicology reviewer concludes that adequate scientific evidence and supporting justification is available or provided to indicate that such constituents are not a concern for cancer risk in the context of ENDS PMTA review.

Tier 1-3 classifications are limited to constituents previously evaluated by either IARC or EPA that have been found by those agencies to demonstrate carcinogenic potential in either human clinical data or within an in vivo experimental system. Toxicology reviewers should not classify constituents that haven't been previously evaluated by EPA or IARC into Tiers 1-3. Constituents included in Tier 1 are carcinogenic

to humans and have a WOE that demonstrates strong evidence of human carcinogenicity. Constituents included in Tier 2 are likely to be carcinogenic to humans and have a WOE that demonstrates carcinogenic potential to humans but does not reach the WOE for the descriptor "Carcinogenic to Humans." Constituents included in Tier 3 have suggestive evidence of carcinogenicity to humans and have a WOE that demonstrates a concern for potential carcinogenic effects in humans, however the available data are judged to be not sufficient for a Tier 1 or Tier 2 classification. Constituents in Tiers 1-3 will be included in the ELCR assessment due to the strength of evidence for carcinogenicity of those constituents, unless, on a case-by-case basis, in alignment with the weight of evidence approach discussed above in "Tiered Weight of Evidence Approach for Carcinogenicity Evaluations," the toxicology reviewer concludes that adequate scientific evidence and supporting justification is available or provided to indicate that such constituents are not a concern for cancer risk in the context of ENDS PMTA review.

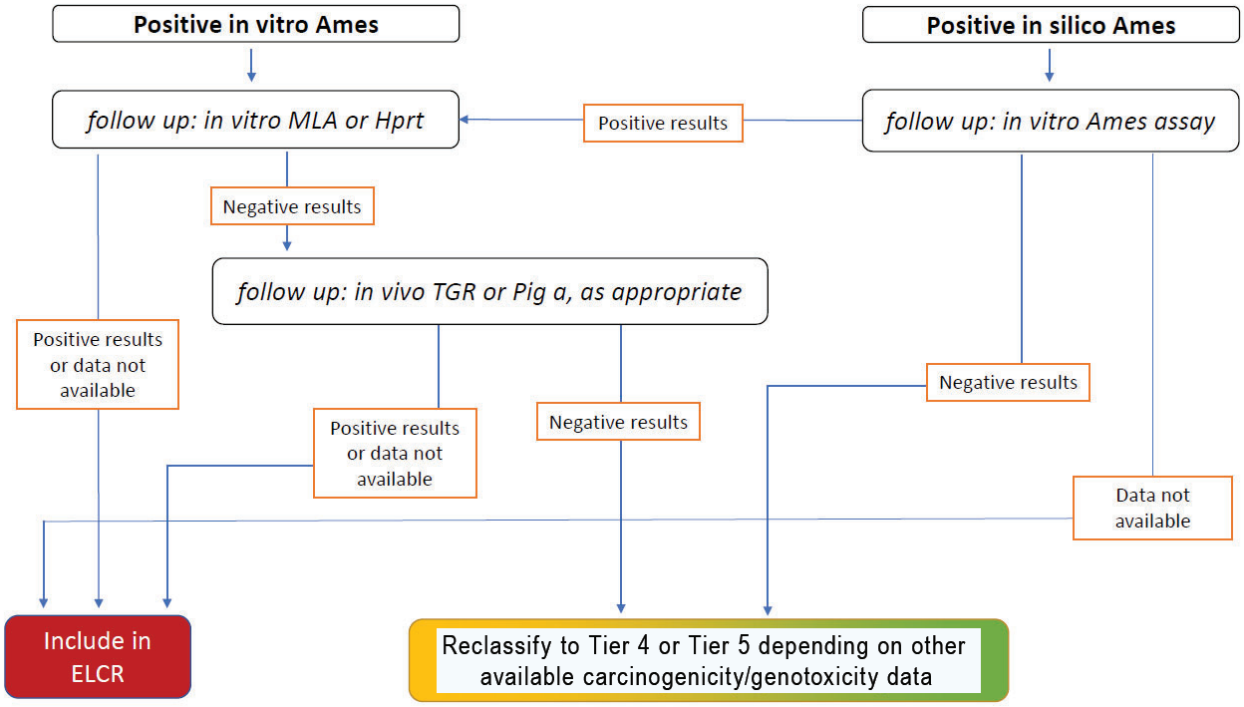
ii. Tier 4A Constituents: A positive carcinogenicity result or a positive in vivo mutagenicity/genotoxicity result



A constituent that has not been formally evaluated by either EPA or IARC, but has scientific data and information indicating that the constituent produced a positive carcinogenicity finding (e.g., increased tumor incidence) in at least one human or animal study, or has evidence of carcinogenicity or genotoxicity in humans or in vivo model systems, will be classified in Tier 4A. Minimally, a positive finding from an in vivo genotoxicity study (e.g., in vivo MN assay) is sufficient for a Tier 4A classification. Tier 4A constituents may have stronger evidence for carcinogenicity beyond these criteria, such as epidemiological evidence of carcinogenicity in humans or extensive evidence of carcinogenicity in animals. A constituent classified in Tier 4A may be included in the ELCR assessment following toxicology review of the study endpoints, protocol, and results. Alternatively, applicants may provide additional scientific evidence and supporting justification in response to CTP communications to evaluate the study endpoints, protocol, and results. Such evidence provided by applicants should be evaluated and considered in the overall toxicology WOE determination. In general, several specific study attributes are considered when evaluating the quality of a study using experimental animals. These attributes include, but are not limited to, test article characterization, dose monitoring, dosing regimen, appropriateness of the experimental animal model, sample sizes, exposure effects on survival and body weight, group

allocation and randomization, histopathological review, data reporting, and data analysis (Samet et al., 2020). If the available information regarding the relevant study endpoints, protocol, and results are determined not to be valid or appropriate for the specific study that was conducted, the constituent will be reclassified according to other existing and available carcinogenicity and genotoxicity data.

iii. Tier 4B Constituents: A positive in vitro or computational Ames mutagenicity assay



Mutagenicity is one aspect of chemical-induced genotoxicity and involves a chemical-induced change in an organism's genetic material. A constituent classified in Tier 4B has a positive finding for mutagenicity from at least one in vitro Ames assay. Alternatively, a constituent that has structural alerts identified by expert-based and statistical-based (Q)SAR models that predict the outcome of an Ames mutagenicity assay would be appropriate for a Tier 4B classification. The Ames mutagenicity assay (OECD TG 471) is highly accurate in predicting the carcinogenicity of test articles (Anderson et al., 1978; Kirkland et al., 2014) and is a widely recognized standard assay to assess the mutagenic potential of a compound. The Ames assay is reported to have a positive predictive value or how often a positive result reflects a true positive, for carcinogenicity of 76-87% (EFSA, 2011; Zeiger, 1998). A constituent having a positive in vitro or computational Ames mutagenicity assay result may be included in the ELCR assessment.

The ICH M7(R2) guidance for assessment and control of DNA reactive impurities in pharmaceuticals indicates that the Ames assay can be used to detect mutagenic carcinogens and limit possible human cancer risk associated with exposure to potentially mutagenic compounds. The use of the Ames assay to assess the mutagenicity of a constituent is also supported in the ICH S2(R1) guidance for genotoxicity testing and data interpretation of pharmaceuticals intended for human use. The ICH M7(R2) guidance provides information on using computational toxicology assessments in hazard identification of mutagenic impurities (ICH, 2023). In the absence of data from the in vitro Ames mutagenicity data, ICH

M7(R2) indicates that a (Q)SAR and SAR computational toxicology assessment may be used as a substitute for in vitro Ames mutagenicity assay data. ICH M7(R2) states that "[t]he absence of structural alerts from two complementary (Q)SAR methodologies (expert-rule based and statistical) is sufficient to conclude that the [constituent] is of no mutagen concern..." ICH M7(R2) further states, "[i]f warranted, the outcome of any computer system-based analysis can be reviewed with the use of expert knowledge in order to provide additional supportive evidence on relevance of any positive, negative, conflicting, or inconclusive prediction and to provide a rationale to support the final conclusion." In light of this guidance, DNCS recommends and supports reviewers evaluating the data from either the in vitro or computational Ames mutagenicity assay to identify mutagenic hazards associated with exposure to an ENDS constituent when available or submitted by the applicant. This mutagenicity data will be used in a WOE approach to assess the overall carcinogenic risk of an ENDS constituent.

For a Tier 4B constituent having a positive outcome from (Q)SAR models for Ames mutagenicity combined with expert review of the computational assessment, an in vitro Ames assay may provide additional information to outweigh the computational assessment. In this scenario, negative results from an in vitro Ames assay may support reclassifying the constituent as Tier 5 if other adequate evidence for a Tier 5 classification is available or reclassifying to a different tier depending on the available carcinogenicity/genotoxicity data. Alternatively, positive findings would support the carcinogenic potential of the constituent and its inclusion in the ELCR assessment.

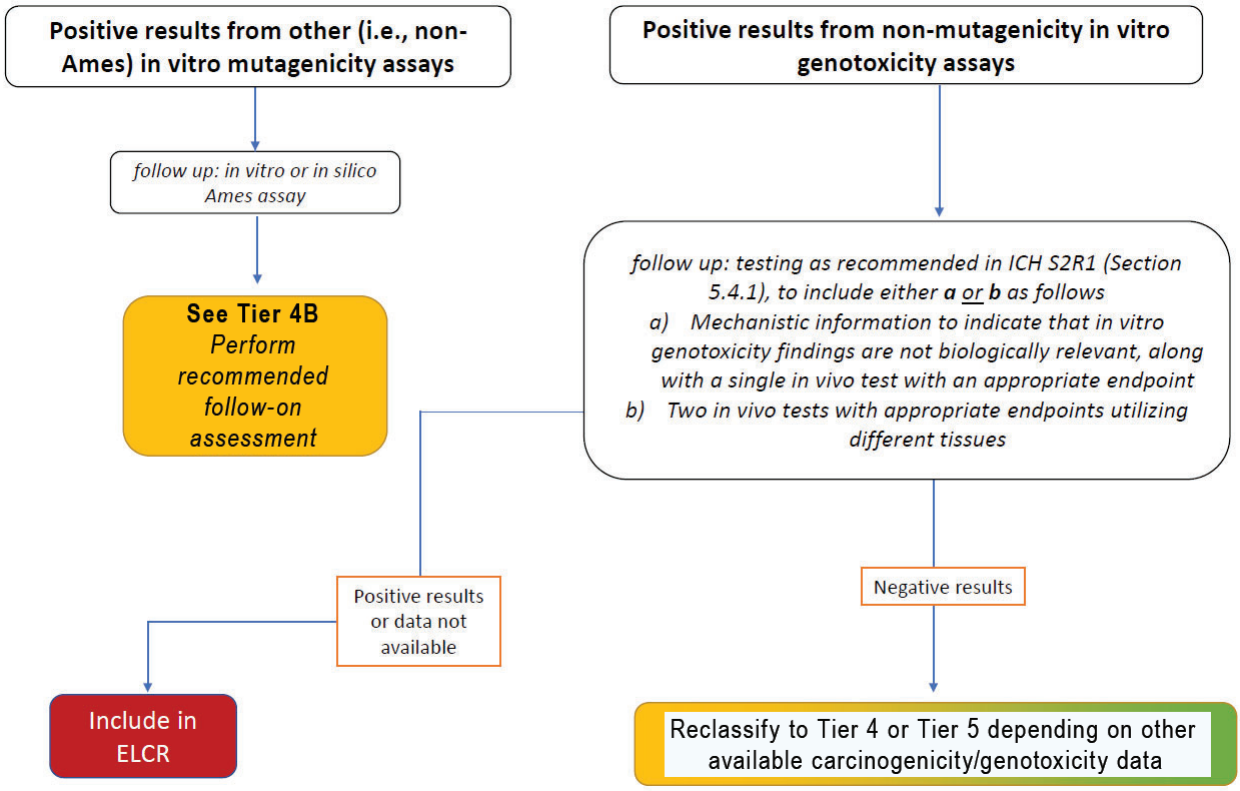
If a constituent is reported to have a positive mutagenicity result from the in vitro Ames assay, the in vitro MLA (OECD TG 490) or mammalian cell hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) gene mutation (OECD TG 476) assays may be used as follow-on assays to further investigate the mutagenic potential of the constituent. For constituents with a positive Ames mutagenicity result, a negative mutagenicity result from either the MLA or *Hprt* gene mutation assay decreased the positive predictive value for carcinogenicity of the Ames assay from 85-92% to 50% (Kirkland et al., 2014). If either the MLA or *Hprt* assays produce a positive result, the constituent should be included in the subsequent ELCR assessment. If either an MLA or *Hprt* follow-on gene mutation assay produces a negative result, negative findings from additional follow-on in vivo transgenic rodent (TGR) (OECD TG 488) and/or in vivo erythrocyte *Pig-a* gene mutation (OECD TG 470) assays may be used to evaluate mutagenicity. As indicated in ICH M7(R2), and further described in Robison et al (Robison et al., 2021), the TGR assay may be used, with justification of target tissue or organ, to investigate the in vivo relevance of any bacterial mutagenicity (i.e., Ames assay) positive result, while the *Pig-a* assay may be an acceptable follow-on assay in certain circumstances. The *Pig-a* assay is appropriate to investigate the in vivo relevance of constituents having a positive Ames assay result without the presence of S9 fraction for metabolic activation (ICH, 2023). If results from either the TGR or *Pig-a* in vivo gene mutation assays are positive for mutagenicity, the constituent will be included in the subsequent ELCR assessment.

If results from in vivo mutagenicity assays are negative, the constituent may be reclassified as Tier 5 if other adequate evidence for a Tier 5 classification is available or to a different tier depending on the available carcinogenicity/genotoxicity data.

Overall, it is expected that negative results in an MLA or *Hprt* in vitro test following a positive Ames would be an infrequent occurrence based upon previously published data (Kirkland et al., 2014).

Therefore, the follow-on in vivo TGR and *Pig-a* mutagenicity assays are anticipated to be infrequently and irregularly needed. In alignment with the Tox21 effort, the inclusion of these follow-on assays is not expected to result in the significant use of in vivo experimental models.

iv. Tier 4C Constituent: Positive results from other (i.e., non-Ames) in vitro genotoxicity assays



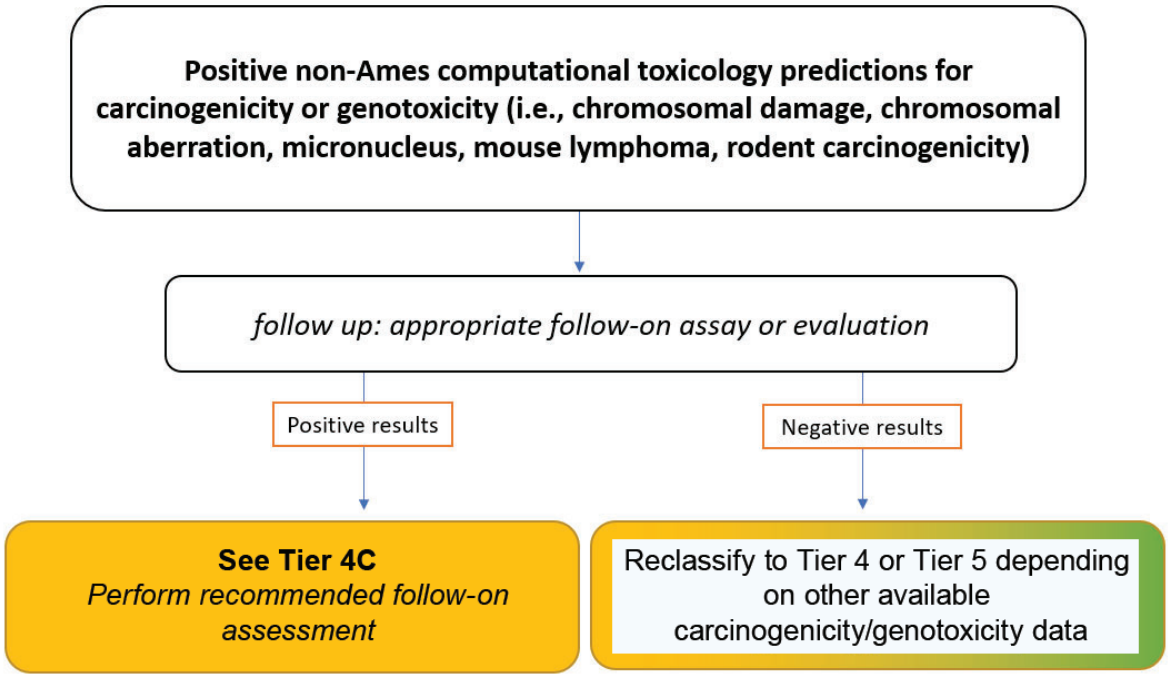
Constituents in Tier 4C have a positive finding from at least one other (i.e., non-Ames) in vitro genotoxicity assay. The ICH S2(R1) guidance for genotoxicity testing and data interpretation of pharmaceuticals intended for human use provides support and a rationale for the use of a battery approach to assess the genotoxicity of a constituent. This guidance indicates that including in vitro mammalian genotoxicity assays in a battery approach together with the Ames assay for mutagenicity increases the sensitivity for detection of rodent carcinogens and broaden the spectrum of genetic events detected, although this approach ultimately decreases the specificity of carcinogenicity predictions. ICH S2(R1) concludes that using a battery approach "is still reasonable because no single test is capable of detecting all genotoxic mechanisms relevant in tumorigenesis."

Many in vitro assays beyond the Ames assay evaluate mutagenicity or other mechanisms of genotoxicity and are regularly used for regulatory evaluations. Relevant examples of such assays include, but are not limited to, the in vitro chromosomal aberration assay, the in vitro MN assay, and the in vitro mouse lymphoma assay, which are identified as appropriate assays to investigate chromosomal damage in ICH S2(R1) (ICH, 2011). The positive predictive values, or how often a positive result reflects a true positive, of these in vitro assays in detecting rodent carcinogens are 67-76%, 76-80%, and 66-74%, respectively (EFSA, 2011). A single positive result from an in vitro NAM is also sufficient for a Tier 4C classification.

A constituent having a positive result from other (i.e., non-Ames) in vitro mutagenicity assays may be included in the ELCR assessment. A follow-on in vitro or computational Ames mutagenicity assay may provide additional information to allay concerns from the positive non-Ames in vitro mutagenicity result depending on the overall WOE, considering factors such as assay predictivity and data quality as evaluated in the toxicology review. In this scenario, available data from a follow-on in vitro or computational Ames mutagenicity assay will cause the constituent to be further evaluated using the Tier 4B decision tree.

A constituent having a positive result from other (i.e., non-Ames) in vitro genotoxicity assays may be included in the ELCR assessment. Alternatively, negative findings from appropriate follow-on assays, as recommended in ICH S2(R1) section 5.4.1, including either a) mechanistic information to indicate that in vitro genotoxicity findings are not biologically relevant, along with a single in vivo test with an appropriate endpoint, or b) two in vivo tests with appropriate endpoints utilizing different tissues may be used to outweigh the reported positive findings from the non-Ames in vitro genotoxicity assays. In this scenario, negative results from the appropriate follow-on assays may support reclassifying the constituent as Tier 5 if other adequate evidence for a Tier 5 classification is available or reclassifying to a different tier depending on the available carcinogenicity/genotoxicity data. Alternatively, positive results would support the potential carcinogenicity of the constituent and its inclusion in the ELCR assessment.

v. Tier 4D constituent: Positive non-Ames computational toxicology predictions for carcinogenicity or genotoxicity



Chemical constituents with positive outcomes from non-Ames computational toxicology assessments for carcinogenicity or genotoxicity are classified as Tier 4D. When empirical data are lacking, computational toxicology evaluations may be used to predict genotoxic hazards for constituents to support regulatory

assessments. Several computational prediction models that evaluate various carcinogenicity, genotoxicity, or mutagenicity outcomes are commercially and freely available. Computational prediction outcomes may be provided by applicants in premarket submissions or performed by DNCS staff in the NCTP team. A constituent having a positive outcome from a non-Ames computational toxicology assessment for rodent carcinogenic potential or genotoxicity may be included in the ELCR assessment.

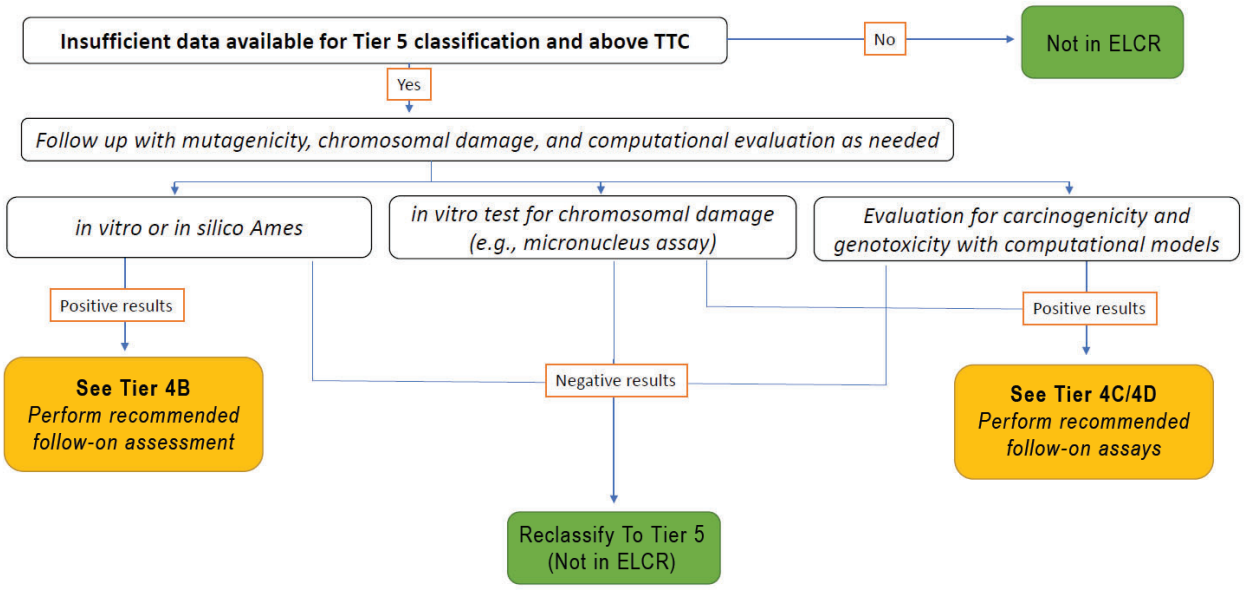
When considering compounds with structural alerts for genotoxic activity, ICH S2R1 indicates that these compounds typically result in positive findings in the standard genotoxicity test batteries. According to ICH S2R1, "negative results in either test battery with a compound that has a structural alert is usually considered sufficient assurance of a lack of genotoxicity (ICH, 2011)." Consistent with this approach and considering the deviations from the standard genotoxicity test batteries in ICH S2R1 as outlined in the 'Tier 5 WOE Considerations' section above, results from both an Ames mutagenicity assay (in vitro or computational) and an assay to assess chromosomal damage may provide sufficient evidence to outweigh genotoxicity concerns from a positive outcome from a non-Ames computational toxicology assessment of a constituent. An appropriate follow-on assay to evaluate chromosomal damage in this scenario would consider the same endpoint of concern identified by the computational prediction. For example, for a constituent classified as Tier 4D with a positive prediction for an in vitro MN assay model, empirical data from an OECD standardized in vitro MN assay may provide additional information to outweigh the positive computational findings. In this scenario, negative results from an in vitro MN assay may support reclassifying the constituent as Tier 5 if other adequate evidence for a Tier 5 classification is available or to a different tier depending on the available carcinogenicity/genotoxicity data. Alternatively, positive results would support the carcinogenic potential of the constituent originally identified computationally. If a positive in vitro result is reported following the computational positive prediction, the constituent may be further assessed using follow-on assays identified in the Tier 4C decision tree.

In the case of positive prediction outcomes from computational assessments using models to detect non-genotoxic carcinogenic potential, additional evidence may be provided by the applicant to further evaluate the predicted outcome. For example, additional evidence could demonstrate that the positive carcinogenicity prediction for a constituent is not relevant to humans (e.g., reported carcinogenic mechanism does not operate in humans), that the potential modes of action (Hernandez et al., 2009) are not applicable for the constituent, or that the exposure is estimated to be at a low level leading to no concern. Additionally, predictions from alternate rodent models that cover non-genotoxic carcinogenicity as a mode of action such as ToxTree (Benigni et al., 2013) or receptor-mediated modes of action (e.g., androgen receptor binding) may alter the weight of evidence to support negative findings overall. Such additional evidence may support reclassifying the constituent as Tier 5 if other adequate evidence for a Tier 5 classification is available or reclassifying to a different tier depending on the available carcinogenicity/genotoxicity data.

In scenarios where applicants did not provide information to outweigh identified concerns for Tier 4D constituents in response to CTP communications, it is feasible that constituents may remain classified in Tier 4D. In this scenario, there is a high level of uncertainty regarding the potential carcinogenicity of such constituents. Toxicology reviewers should determine whether including such constituents in the cumulative ELCR ($ELCR_c$) calculations would affect either the qualitative risk management descriptor of

the product under review relative to 1R6F cigarettes or the relationship relative to the median of the ENDS MGO marketplace as outlined in the companion DNCS memorandum (see Memorandum: Calculating Excess Lifetime Cancer Risk in ENDS Tobacco Product Applications, June 3, 2024). If including these Tier 4D constituents in the $ELCR_c$ changes the qualitative risk management descriptor of the ENDS under review relative to the 1R6F cigarette or the relationship relative to the median of the ENDS MGO marketplace, there is a high level of uncertainty regarding the overall cancer risk of such products, and toxicology reviewers should refrain from making a final $ELCR_c$ calculation for such products due to this high level of uncertainty. In this scenario, toxicology discipline reviews should highlight the lack of sufficient information (e.g., empirical test data) to adequately address carcinogenic hazards for the product under review. Conversely, if including these Tier 4D constituents in the $ELCR_c$ does not change the qualitative risk management descriptor of the ENDS under review relative to the 1R6F cigarette or the relationship relative to the median of the ENDS MGO marketplace, toxicology reviewers should proceed with calculating a final $ELCR_c$ that does not include such Tier 4D constituents, since additional information to clarify the uncertainty for these Tier 4D constituents is unlikely to change our overall cancer risk evaluation of products in this scenario. Toxicology reviewers should convey this information in their review as part of the risk characterization.

vi. Tier 4E Constituent: Insufficient data available for Tier 5 classification



Constituents classified in Tier 4E lack sufficient data for a Tier 5 classification and do not fit into any other tiers outlined herein. If insufficient data are available to classify a constituent as Tier 5, but the constituent is below the relevant threshold of toxicological concern (TTC) as identified and described in a separate DNCS memorandum (see Memorandum: Calculating Excess Lifetime Cancer Risk in ENDS Tobacco Product Applications, June 3, 2024), the constituent will be excluded from the ELCR assessment. Notably, as suggested by Kroes et al., (Kroes et al., 2004) a TTC approach is not suitable for certain high potency chemicals (e.g., aflatoxin-like, N-nitroso, azoxy- compounds), including metals; therefore, this TTC would not apply to such constituents.

If insufficient data are available to classify a constituent as Tier 5, but the constituent is above the relevant TTC as identified and described in a separate DNCS memorandum (see Memorandum: Calculating Excess Lifetime Cancer Risk in ENDS Tobacco Product Applications, June 3, 2024), additional information regarding the constituent's mutagenicity, genotoxicity, and carcinogenicity, as needed, may support reclassifying the constituent. Specific examples are as follows:

- Following a positive result from an in vitro or computational Ames mutagenicity assay, the constituent will be reclassified as Tier 4B and additional information, as described in the Tier 4B decision tree, may support reclassifying the constituent.
- Following a positive result from an in vitro test for chromosomal damage (e.g., MN assay), the constituent will be reclassified as Tier 4C and additional information, as described in the Tier 4C decision tree, may support reclassifying the constituent.
- Following a positive result from a computational non-Ames computational toxicology evaluation, the constituent will be reclassified as Tier 4D and additional information, as described in the Tier 4D decision tree, may support reclassifying the constituent.
- For a Tier 4E constituent with a negative in vitro MN assay but no available data regarding the mutagenicity of the constituent, negative prediction outcomes from a computational Ames assessment using both expert-rule based and a statistical-based QSAR methodology may support reclassifying the constituent as Tier 5 in the absence of other positive or equivocal data elsewhere (e.g., in vivo, in vitro, or computational data).
- If the corresponding in vitro mutagenicity, in vitro genotoxicity, and computational toxicology data yield negative results, the constituent may be classified as Tier 5, dependent on the WOE for human carcinogenic risk. If the constituent is classified as Tier 5, the constituent will not be included in the ELCR.

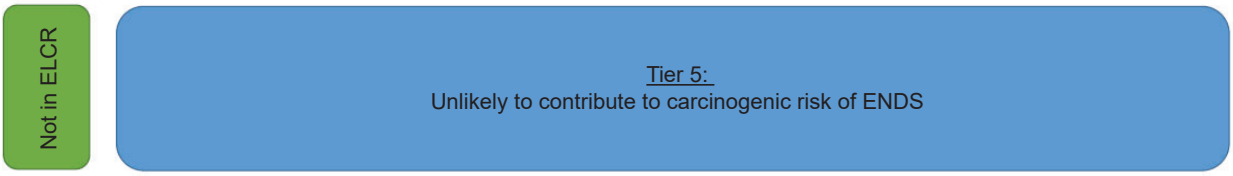
In scenarios where applicants did not provide information to adequately address a constituent's mutagenicity, genotoxicity, and carcinogenicity in response to CTP communications, it is feasible that constituents may remain classified in Tier 4E. Whether or not a chemical structure is available for such constituents will affect the ability to perform hazard identification and, therefore, the overall certainty regarding the genotoxicity or carcinogenicity of such constituents. Toxicology reviewers should take the following approaches in such scenarios:

- For Tier 4E constituents above the TTC where it is not feasible for further assessment to be performed and no further data are available (e.g., unidentified leachable with unknown structure for which an applicant did not provide any additional information in response to CTP communications), such constituents will remain in Tier 4E. In this scenario, there a high level of uncertainty regarding the potential carcinogenicity of such constituents. Toxicology reviewers should determine whether including such constituents in the $ELCR_c$ calculations would affect either the qualitative risk management descriptor of the product under review relative to 1R6F cigarettes or the relationship relative to the median of the ENDS MGO marketplace as outlined in the companion DNCS memorandum (see Memorandum: Calculating Excess Lifetime Cancer Risk in ENDS Tobacco Product Applications, June 3, 2024). If including these Tier 4E constituents in the $ELCR_c$ changes the qualitative risk management descriptor of the ENDS under review relative to the 1R6F cigarette or the relationship relative to the median of the ENDS MGO

marketplace, there is a high level of uncertainty regarding the overall cancer risk of such products, and toxicology reviewers should refrain from making an $ELCR_c$ calculation for such products due to this high level of uncertainty. In this scenario, toxicology discipline reviews should highlight the lack of sufficient information (e.g., constituent identity) to adequately address carcinogenic hazards for the product under review. Conversely, if including these Tier 4E constituents in the $ELCR_c$ does not change the qualitative risk management descriptor of the ENDS under review relative to the 1R6F cigarette or the relationship relative to the median of the ENDS MGO marketplace, toxicology reviewers should proceed with calculating an $ELCR_c$ that does not include such Tier 4E constituents, since additional information to clarify the uncertainty for these Tier 4E constituents is unlikely to change our overall cancer risk evaluation of products in this scenario. Toxicology reviewers should convey this information in their review as part of the risk characterization.

- It is feasible that constituents with a known chemical structure may remain classified in Tier 4E if applicants did not provide information to adequately address a constituent's mutagenicity, genotoxicity, and carcinogenicity in response to CTP communications. Such Tier 4E constituents may not have sufficient information available for a Tier 5 classification but may have an overall WOE that leans towards negative findings for genotoxicity and carcinogenicity. For example, a constituent may have a negative prediction from a computational Ames assay and no positive or equivocal predicted outcomes from computational toxicology evaluations but lack an in vitro or in vivo study to assess chromosomal damage. Such a constituent would only meet 2 out of 3 criteria for a Tier 5 classification and would therefore remain classified as Tier 4E. Although the overall weight of evidence in such scenarios leans towards negative, there is some uncertainty about a negative overall conclusion due to the lack of in vitro or in vivo data to assess chromosomal damage. For Tier 4E constituents that cannot be reclassified to other tiers but have an overall WOE that leans towards negative, toxicology reviewers should calculate an $ELCR_c$ excluding such Tier 4E constituents, for the product under review. However, in this scenario, toxicology reviewers should include language in the key findings of their discipline reviews to communicate the uncertainty for the Tier 4E constituents by describing the full WOE available, what information is lacking for a Tier 5 classification, and indicating how the $ELCR_c$ calculation, qualitative risk management descriptor relative to 1R6F cigarettes, and relation to the MGO ENDS marketplace would change if, in the worst case scenario, such constituents are actually carcinogens and were included in the $ELCR_c$ calculation.

vii. Tier 5 Constituent: Unlikely to contribute to carcinogenic risk of ENDS



Constituents in Tier 5 are unlikely to contribute to the carcinogenic risk of ENDS. Tier 5 constituents have available data that are robust for deciding that there is no basis for a genotoxicity and/or carcinogenic concern in the context of ENDS premarket application review and are not included in an ELCR

assessment. Constituents classified as EPA Group E will be placed in Tier 5. For constituents with carcinogenicity studies available, evidence from well-designed and well-conducted animal studies that demonstrate a *lack* of carcinogenic effect via the inhalation route, in the absence of other animal or human data suggesting a potential for carcinogenic effects, are appropriate for inclusion in Tier 5. When carcinogenicity data are not available for a constituent, a Tier 5 classification may be appropriate based upon genotoxicity evidence alone. As adapted from ICH S2(R1), adequate genotoxicity evidence includes all of the following if the WOE does not identify conflicting positive or equivocal results elsewhere:

- One negative finding for mutagenicity from either an in vitro, in vivo, or computational test, and
- One negative finding for chromosomal damage from either an in vitro, or in vivo test, and
- No positive or equivocal predicted outcomes from computational toxicology evaluations.

Furthermore, as identified and described in FDA Memorandum: Calculating Excess Lifetime Cancer Risk in Tobacco Product Applications (June 3, 2024), data-limited constituents present at levels below the relevant TTC will be excluded from the ELCR assessment. Notably, as suggested by Kroes et al. (Kroes et al., 2004), the TTC approach is not designed to replace conventional risk characterization approaches for established and well-studied chemicals. As discussed in Kroes et al., and Serafimova et al., ((Serafimova et al., 2021)) a TTC approach would not normally be applied to inorganic chemicals, heavy metals, proteins, steroids, nanomaterials, radioactive chemicals, organosilicon chemicals, chemicals with potential for bioaccumulation (e.g., polyhalogenated-dibenzodioxins, -dibenzofurans, and -biphenyls), and high-potency carcinogens (e.g., aflatoxin-like, N-nitroso, azoxy- compounds).

Tables

Table 1: IARC Monographs Definitions of Weight of evidence Descriptors for the Evidence Streams
[adapted from Samet et al 2020: JNCI J Natl Cancer Inst (2020) 112(1): djz169]

Weight-of-Evidence Descriptor	Cancer in Humans	Cancer in Experimental Animals	Mechanistic Evidence
Sufficient (or strong for mechanistic evidence)	A causal association has been established: A positive association has been observed in the body of evidence on exposure to the agent and cancer in studies in which chance, bias, and confounding were ruled out with reasonable confidence.	A causal relationship has been established between exposure to the agent and cancer in experimental animals based on an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals, (b) two or more independent studies in one species carried out at different times or in different laboratories and/or under different protocols. or (c) in both sexes of a single species in a well-conducted study.	Results in several different experimental systems are consistent, and the overall mechanistic database is coherent. Further support can be provided by studies that demonstrate experimentally that the suppression of key mechanistic processes leads to the suppression of tumor development. Typically, a substantial number of studies on a range of relevant endpoints are available in one or more mammalian species.*
Limited	A causal interpretation of the positive association observed in the body of evidence on exposure to the agent and cancer is credible, but chance, bias, or confounding could not be ruled out with reasonable confidence.	The data suggest a carcinogenic effect but are limited for making a definitive evaluation because, for example, (a) evidence of carcinogenicity is restricted to a single experiment; (b) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; (c) the agent increases tumor multiplicity or decreases tumor latency but does not increase tumor incidence; and (d) the evidence of carcinogenicity is restricted to initiation–promotion studies.	The evidence is suggestive, but, for example, (a) the studies cover a narrow range of experiments, relevant endpoints, and/or species; (b) there are unexplained inconsistencies in studies of similar design; and/or (c) there is unexplained incoherence across studies of different endpoints or in different experimental systems.

Weight-of-Evidence Descriptor	Cancer in Humans	Cancer in Experimental Animals	Mechanistic Evidence
Inadequate	No data are available, or the available studies are of insufficient quality, consistency, or statistical precision to permit a conclusion to be drawn about the presence or the absence of a causal association between exposure and cancer.	The studies cannot be interpreted as showing either the presence or the absence of a carcinogenic effect because of major qualitative or quantitative limitations, or no data are available on cancer in experimental animals.	Few or no data are available; there are unresolved questions about the adequacy of the design, conduct, or interpretation of the studies; and/or the available results are negative.

*Quantitative structure–activity considerations, in vitro tests in nonhuman mammalian cells, and experiments in nonmammalian species may provide corroborating evidence but typically do not in themselves provide strong evidence. However, consistent findings across a number of different test systems in different species may provide strong evidence.

Table 2: IARC Monographs Integration of Streams of Evidence in Reaching Overall Classifications
[adapted from Samet et al 2020: JNCI J Natl Cancer Inst (2020) 112(1): djz169]

Cancer in Humans*	Cancer in Experimental Animals	Mechanistic Evidence	Basis of Overall Evaluation	Classification Based on Strength of Evidence
Sufficient	Not Necessary	Not Necessary	Cancer in Humans	**Carcinogenic to Humans (Group 1)**
Limited or Inadequate	Sufficient	Strong: Key Characteristics of Carcinogens, from exposed humans	Cancer in Experimental Animals and Mechanistic Evidence	
Limited	Sufficient	Not Necessary	Cancer in Humans and Cancer in Experimental Animals	**Probably Carcinogenic to Humans (Group 2A)**
Inadequate	Sufficient	Strong: Key Characteristics of Carcinogens, From Human Cells or Tissues	Cancer in Experimental Animals and Mechanistic Evidence	
Limited	Less Than Sufficient	Strong: Key Characteristics of Carcinogens	Cancer in Humans and Mechanistic Evidence	
Limited or Inadequate	Not Necessary	Strong: The Agent Belongs to a Mechanistic Class of Agents for Which One or More Members Have Been Classified in Group 2A or 1	Mechanistic Evidence	
Limited	Less Than Sufficient	Limited or Inadequate	Cancer in Humans	**Possibly Carcinogenic to Humans (Group 2B)**
Inadequate	Sufficient	Not Necessary	Cancer in Experimental Animals	
Inadequate	Less Than Sufficient	Strong: Key Characteristics of Carcinogens	Mechanistic Evidence	

Limited	Sufficient	Strong: The Mechanism of Carcinogenicity in Experimental Animals Does Not Operate in Humans†	Cancer in Humans and Mechanistic Evidence	
Inadequate	Sufficient	Strong: The Mechanism of Carcinogenicity in Experimental Animals Does Not Operate in Humans†	Mechanistic Evidence	**Not Classifiable as to its Carcinogenicity to Humans (Group 3)**
All Other Situations Not Listed Above				

* Highest strength of evidence for any cancer site(s)

† The "strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans" must specifically be for the tumor sites supporting the classification of "sufficient evidence in experimental animals."

Table 3: EPA Weight of Evidence Narrative and Carcinogenicity Classifications

[adapted from Guidelines for Carcinogen Risk Assessment, EPA 2005]

"Carcinogenic to Humans"
Indicates strong evidence of human carcinogenicity
This descriptor is appropriate when there is convincing epidemiological evidence of a causal association between human exposure and cancer
This descriptor may be equally appropriate with a lesser weight of epidemiologic evidence that is strengthened by other lines of evidence. It can be used when **all of** the following conditions are met: (a) there is strong evidence of an association between human exposure and either cancer or the key precursor events of the agent's mode of action but not enough for a causal association, and (b) there is extensive evidence of carcinogenicity in animals, and (c) the mode(s) of carcinogenic action and associated key precursor events have been identified in animals, and (d) there is strong evidence that the key precursor events that precede the cancer response in animals are anticipated to occur in humans and progress to tumors, based on available biological information.

"Likely to be Carcinogenic to Humans"
This descriptor is appropriate when the weight of evidence is adequate to demonstrate carcinogenic potential to humans **but does not** reach the weight of evidence for the descriptor "Carcinogenic to Humans." Supporting data for this descriptor may include: • An agent demonstrating a plausible (but not definitively causal) association between human exposure and cancer, in most cases with some supporting biological, experimental evidence, though not necessarily carcinogenicity data from animal experiments; • An agent that has tested positive in animal experiments in more than one species, sex, strain, site, or exposure route, with or without evidence of carcinogenicity in humans; • A positive tumor study that raises additional biological concerns beyond that of a statistically significant result, for example, a high degree of malignancy, or an early age at onset; • A rare animal tumor response in a single experiment that is assumed to be relevant to humans; or • A positive tumor study that is strengthened by other lines of evidence, for example, either plausible (but not definitively causal) association between human exposure and cancer or evidence that the agent or an important metabolite causes events generally known to be associated with tumor formation (such as DNA reactivity or effects on cell growth control) likely to be related to the tumor response in this case.

"Suggestive Evidence of Carcinogenic Potential"
This descriptor is appropriate when the weight of evidence is suggestive of carcinogenicity; a concern for potential carcinogenic effects in humans is raised, but the data are judged not sufficient for a stronger conclusion.
This descriptor covers a spectrum of evidence associated with varying levels of concern for carcinogenicity, ranging from a positive cancer result in the only study on an agent to a single positive cancer result in an extensive database that includes negative studies in other species. Depending on the extent of the database, additional studies may or may not provide further insights. Some examples include: • A small, and possibly not statistically significant, increase in tumor incidence observed in a single animal or human study that does not reach the weight of evidence for the descriptor "Likely to Be Carcinogenic to Humans." The study generally would not be contradicted by other studies of equal quality in the same population group or experimental system (see discussions of *conflicting evidence* and *differing results*, below); • A small increase in a tumor with a high background rate in that sex and strain, when there is some but insufficient evidence that the observed tumors may be due to intrinsic factors that cause background tumors and not due to the agent being assessed. (When there is a high background rate of a specific tumor in animals of a particular sex and strain, then there may be biological factors operating

"Suggestive Evidence of Carcinogenic Potential"
independently of the agent being assessed that could be responsible for the development of the observed tumors.) In this case, the reasons for determining that the tumors are not due to the agent are explained; • Evidence of a positive response in a study whose power, design, or conduct limits the ability to draw a confident conclusion (but does not make the study fatally flawed), but where the carcinogenic potential is strengthened by other lines of evidence (such as structure-activity relationships); or • A statistically significant increase at one dose only, but no significant response at the other doses and no overall trend.

"Inadequate Evidence of Carcinogenic Potential"
This descriptor is appropriate when the available evidence is judged inadequate for applying one of the other descriptors.
Additional studies generally would be expected to provide further insights. Some examples include: • Little or no pertinent information; • Conflicting evidence, that is, some studies provide evidence of carcinogenicity but other studies of equal quality in the same sex and strain are negative. *Differing results*, that is, positive results in some studies and negative results in one or more different experimental systems, do not constitute *conflicting evidence,* as the term is used here. Depending on the overall weight of evidence, differing results can be considered either suggestive evidence or likely evidence; or • Negative results that are not sufficiently robust for the descriptor, "Not Likely to Be Carcinogenic to Humans."

"Not Likely to be Carcinogenic to Humans"
This descriptor is appropriate when the available data considered robust for deciding that there is no basis for human hazard concern. A descriptor of "not likely" applies only to the circumstances supported by the data. For example, an agent may be "Not Likely to Be Carcinogenic" by one route but not necessarily by another. In those cases that have positive animal experiment(s) but the results are judged to be not relevant to humans, the narrative discusses why the results are not relevant.
In some instances, there can be positive results in experimental animals when there is strong, consistent evidence that each mode of action in experimental animals does not operate in humans. In other cases, there can be convincing evidence in both humans and animals that the agent is not carcinogenic. The judgment may be based on data such as: • Animal evidence that demonstrates lack of carcinogenic effect in both sexes in well-designed and well-conducted studies in at least two appropriate animal species (in the absence of other animal or human data suggesting a potential for cancer effects), • Convincing and extensive experimental evidence showing that the only carcinogenic effects observed in animals are not relevant to humans, • Convincing evidence that carcinogenic effects are not likely by a particular exposure route, or • Convincing evidence that carcinogenic effects are not likely below a defined dose range.

References

Anderson D, Styles JA. Appendix ii. The bacterial mutation test. *British Journal of Cancer*. 1978/06/01 1978;37(6):924-930. doi:10.1038/bjc.1978.134

Bartsch H, Malaveille C. Prevalence of genotoxic chemicals among animal and human carcinogens evaluated in the iarc monograph series. *Cell Biology and Toxicology*. 1989/06/01 1989;5(2):115-127. doi:10.1007/BF00122647

Behar RZ, Luo W, McWhirter KJ, Pankow JF, Talbot P. Analytical and toxicological evaluation of flavor chemicals in electronic cigarette refill fluids. *Sci Rep*. May 29 2018;8(1):8288. doi:10.1038/s41598-018-25575-6

Benigni R, Bossa C, Tcheremenskaia O. Nongenotoxic carcinogenicity of chemicals: Mechanisms of action and early recognition through a new set of structural alerts. *Chem Rev*. May 8 2013;113(5):2940-57. doi:10.1021/cr300206t

Beronius A, Zilliacus J, Hanberg A, Luijten M, van der Voet H, van Klaveren J. Methodology for health risk assessment of combined exposures to multiple chemicals. *Food Chem Toxicol*. Sep 2020;143:111520. doi:10.1016/j.fct.2020.111520

Bopp SK, Barouki R, Brack W, Dalla Costa S, Dorne J-LCM, Drakvik PE, Faust M, Karjalainen TK, Kephalopoulos S, van Klaveren J, Kolossa-Gehring M, Kortenkamp A, Lebret E, Lettieri T, Nørager S, Rüegg J, Tarazona JV, Trier X, van de Water B, . . . Bergman Å. Current eu research activities on combined exposure to multiple chemicals. *Environment International*. 2018/11/01/ 2018;120:544-562. doi:https://doi.org/10.1016/j.envint.2018.07.037

COM. *Uk committee on mutagenicity of chemicals in food, consumer products and the environment (com): Guidance on a strategy for genotoxicity testing of chemicals*. 2021. https://www.gov.uk/government/publications/a-strategy-for-testing-of-chemicals-for-genotoxicity

EC. *Communication from the comission: The combination effects of chemicals chemical mixtures*. 2012. https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX:52012DC0252

EFSA. Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. *EFSA Journal*. 2011;9(9):2379. doi:10.2903/j.efsa.2011.2379

EFSA, Hardy A, Benford D, Halldorsson T, Jeger M, Knutsen HK, More S, Naegeli H, Noteborn H, Ockleford C, Ricci A, Rychen G, Silano V, Solecki R, Turck D, Younes M, Aquilina G, Crebelli R, Gürtler R, . . . Schlatter J. Clarification of some aspects related to genotoxicity assessment. *EFSA Journal*. 2017;15(12):e05113. doi:https://doi.org/10.2903/j.efsa.2017.5113

EFSA, More S, Bampidis V, Benford D, Boesten J, Bragard C, Halldorsson T, Hernandez-Jerez A, Hougaard-Bennekou S, Koutsoumanis K, Naegeli H, Nielsen SS, Schrenk D, Silano V, Turck D, Younes M, Aquilina G, Crebelli R, Gürtler R, . . . Schlatter J. Genotoxicity assessment of chemical mixtures. https://doi.org/10.2903/j.efsa.2019.5519. *EFSA Journal*. 2019/01/01 2019;17(1):e05519. doi:https://doi.org/10.2903/j.efsa.2019.5519

Escher BI, Stapleton HM, Schymanski EL. Tracking complex mixtures of chemicals in our changing environment. *Science*. 2020;367(6476):388-392. doi:doi:10.1126/science.aay6636

Farsalinos KE, Gillman G. Carbonyl emissions in e-cigarette aerosol: A systematic review and methodological considerations. *Front Physiol*. 2017;8:1119. doi:10.3389/fphys.2017.01119

Fortin A-MV, Long AS, Williams A, Meier MJ, Cox J, Pinsonnault C, Yauk CL, White PA. Application of a new approach methodology (nam)-based strategy for genotoxicity assessment of data-poor compounds. Original Research. *Frontiers in Toxicology*. 2023-January-23 2023;5doi:10.3389/ftox.2023.1098432

Gaylor DW. The use of haber's law in standard setting and risk assessment. *Toxicology*. 2000/08/14/ 2000;149(1):17-19. doi:https://doi.org/10.1016/S0300-483X(00)00228-6

Gray N, Halstead M, Valentin-Blasini L, Watson C, Pappas RS. Toxic metals in liquid and aerosol from pod-type electronic cigarettes. *J Anal Toxicol*. Feb 14 2022;46(1):69-75. doi:10.1093/jat/bkaa185

Halstead M, Gray N, Gonzalez-Jimenez N, Fresquez M, Valentin-Blasini L, Watson C, Pappas RS. Analysis of toxic metals in electronic cigarette aerosols using a novel trap design. *J Anal Toxicol*. Mar 7 2020;44(2):149-155. doi:10.1093/jat/bkz078

Hernandez LG, van Steeg H, Luijten M, van Benthem J. Mechanisms of non-genotoxic carcinogens and importance of a weight of evidence approach. *Mutat Res*. Sep-Dec 2009;682(2-3):94-109. doi:10.1016/j.mrrev.2009.07.002

IARC. *Dry cleaning, some chlorinated solvents and other industrial chemicals*. Vol. 63. 1995. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* https://publications.iarc.fr/81

IARC. *Preamble to the iarc monographs*. 2019a. https://monographs.iarc.who.int/wp-content/uploads/2019/07/Preamble-2019.pdf

IARC. *Tumour site concordance and mechanisms of carcinogenesis: Iarc scientific publication no. 165*. 2019b.

IARC. *Acrolein, crotonaldehyde, and arecoline*. Vol. 128. 2021. *IARC Monographs on the Evaluation of Carcinogenic Hazards to Humans* https://publications.iarc.fr/602

ICCVAM. *Interagency coordinating committee on the validation of alternative methods: Validation, qualification, and regulatory acceptance of new approach methodologies*. (2024). Retrieved from https://ntp.niehs.nih.gov/sites/default/files/2024-03/VWG_Report_27Feb2024_FD_508.pdf

ICH. *S2(r1) guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use*. (2011). Retrieved from https://www.ema.europa.eu/en/ich-s2-r1-genotoxicity-testing-data-interpretation-pharmaceuticals-intended-human-use-scientific

ICH. *M7(r2) assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk*. (2023). Retrieved from https://www.ema.europa.eu/en/ich-m7-assessment-control-dna-reactive-mutagenic-impurities-pharmaceuticals-limit-potential

Jabba SV, Diaz AN, Erythropel HC, Zimmerman JB, Jordt SE. Chemical adducts of reactive flavor aldehydes formed in e-cigarette liquids are cytotoxic and inhibit mitochondrial function in respiratory epithelial cells. *Nicotine Tob Res*. Dec 15 2020;22(Suppl 1):S25-S34. doi:10.1093/ntr/ntaa185

Judson R, Houck K, Martin M, Richard AM, Knudsen TB, Shah I, Little S, Wambaugh J, Woodrow Setzer R, Kothya P, Phuong J, Filer D, Smith D, Reif D, Rotroff D, Kleinstreuer N, Sipes N, Xia M, Huang R, . . .

Thomas RS. Editor's highlight: Analysis of the effects of cell stress and cytotoxicity on in vitro assay activity across a diverse chemical and assay space. *Toxicological Sciences*. 2016;152(2):323-339. doi:10.1093/toxsci/kfw092

Kavlock RJ, Bahadori T, Barton-Maclaren TS, Gwinn MR, Rasenberg M, Thomas RS. Accelerating the pace of chemical risk assessment. *Chemical Research in Toxicology*. 2018/05/21 2018;31(5):287-290. doi:10.1021/acs.chemrestox.7b00339

Kenyon MO, Cheung JR, Dobo KL, Ku WW. An evaluation of the sensitivity of the ames assay to discern low-level mutagenic impurities. *Regulatory Toxicology and Pharmacology*. 2007/06/01/ 2007;48(1):75-86. doi:https://doi.org/10.1016/j.yrtph.2007.01.006

Kienzler A, Berggren E, Bessems J, Bopp S, Linden S, Worth A. *Assessment of mixtures : Review of regulatory requirements and guidance*. Publications Office of the European Union; 2017.

Kienzler A, Bopp SK, van der Linden S, Berggren E, Worth A. Regulatory assessment of chemical mixtures: Requirements, current approaches and future perspectives. *Regul Toxicol Pharmacol*. Oct 2016;80:321-34. doi:10.1016/j.yrtph.2016.05.020

Kirkland D, Zeiger E, Madia F, Corvi R. Can in vitro mammalian cell genotoxicity test results be used to complement positive results in the ames test and help predict carcinogenic or in vivo genotoxic activity? Ii. Construction and analysis of a consolidated database. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 2014/12/01/ 2014;775-776:69-80. doi:https://doi.org/10.1016/j.mrgentox.2014.10.006

Kroes R, Renwick AG, Cheeseman M, Kleiner J, Mangelsdorf I, Piersma A, Schilter B, Schlatter J, van Schothorst F, Vos JG, Wurtzen G, European branch of the International Life Sciences I. Structure-based thresholds of toxicological concern (ttc): Guidance for application to substances present at low levels in the diet. *Food Chem Toxicol*. Jan 2004;42(1):65-83. doi:10.1016/j.fct.2003.08.006

Krusemann EJZ, Havermans A, Pennings JLA, de Graaf K, Boesveldt S, Talhout R. Comprehensive overview of common e-liquid ingredients and how they can be used to predict an e-liquid's flavour category. *Tob Control*. Mar 2021;30(2):185-191. doi:10.1136/tobaccocontrol-2019-055447

Kumari M, Kumar A. Identification of component-based approach for prediction of joint chemical mixture toxicity risk assessment with respect to human health: A critical review. *Food and Chemical Toxicology*. 2020/09/01/ 2020;143:111458. doi:https://doi.org/10.1016/j.fct.2020.111458

Lee H-W, Park S-H, Weng M-w, Wang H-T, Huang WC, Lepor H, Wu X-R, Chen L-C, Tang M-s. E-cigarette smoke damages DNA and reduces repair activity in mouse lung, heart, and bladder as well as in human lung and bladder cells. *Proceedings of the National Academy of Sciences*. 2018;115(7):E1560-E1569. doi:doi:10.1073/pnas.1718185115

Misra M, Leverette RD, Cooper BT, Bennett MB, Brown SE. Comparative in vitro toxicity profile of electronic and tobacco cigarettes, smokeless tobacco and nicotine replacement therapy products: E-liquids, extracts and collected aerosols. *International Journal of Environmental Research and Public Health*. 2014;11(11):11325-11347. https://www.mdpi.com/1660-4601/11/11/11325

Norwood DL, Paskiet D, Ruberto M, Feinberg T, Schroeder A, Poochikian G, Wang Q, Deng TJ, DeGrazio F, Munos MK, Nagao LM. Best practices for extractables and leachables in orally inhaled and nasal drug products: An overview of the pqri recommendations. *Pharm Res*. Apr 2008;25(4):727-39. doi:10.1007/s11095-007-9521-z

OECD. *Guidance document 116 on the conduct and design of chronic toxicity and carcinogenicity studies, supporting test guidelines 451, 452 and 453*. 2014.

OECD. *Considerations for assessing the risks of combined exposure to multiple chemicals*. 2018. https://www.oecd-ilibrary.org/content/publication/ceca15a9-en

Robison TW, Heflich RH, Manjanatha MG, Elespuru R, Atrakchi A, Mei N, Ding W. Appropriate in vivo follow-up assays to an in vitro bacterial reverse mutation (ames) test positive investigational drug candidate (active pharmaceutical ingredient), drug-related metabolite, or drug-related impurity. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 2021/08/01/ 2021;868-869:503386. doi:https://doi.org/10.1016/j.mrgentox.2021.503386

Samet JM, Chiu WA, Cogliano V, Jinot J, Kriebel D, Lunn RM, Beland FA, Bero L, Browne P, Fritschi L, Kanno J, Lachenmeier DW, Lan Q, Lasfargues G, Le Curieux F, Peters S, Shubat P, Sone H, White MC, . . . Wild CP. The iarc monographs: Updated procedures for modern and transparent evidence synthesis in cancer hazard identification. *J Natl Cancer Inst*. Jan 1 2020;112(1):30-37. doi:10.1093/jnci/djz169

Serafimova R, Coja T, Kass GEN. Application of the threshold of toxicological concern (ttc) in food safety: Challenges and opportunities. *Front Toxicol*. 2021;3:655951. doi:10.3389/ftox.2021.655951

USEPA. *Supplementary guidance for conducting health risk assessment of chemical mixtures*. 2000:1-209. EPA/630/R-00/002. http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=20533

USEPA. *Guidelines for carcinogen risk assessment*. EPA Report. 2005. EPA630P03001F. https://www.epa.gov/sites/production/files/2013-09/documents/cancer_guidelines_final_3-25-05.pdf

USEPA. *Concepts, methods, and data sources for cumulative health risk assessment of multiple chemicals, exposures, and effects: A resource document*. EPA Report. 2007. EPA/600/R-06/013F. http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=190187

USFDA. *Harmful and potentially harmful constituents in tobacco products and tobacco smoke; established list.* Federal Registry (2012).

USFDA. *Harmful and potentially harmful constituents in tobacco products and tobacco smoke; established list; proposed additions*. Federal Registry (2019).

USFDA. Premarket tobacco product applications for electronic nicotine delivery systems (ends) guidance for industry. 2023:41. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/premarket-tobacco-product-applications-electronic-nicotine-delivery-systems-ends

Waters MD, Stack HF, Jackson MA. Genetic toxicology data in the evaluation of potential human environmental carcinogens. *Mutation Research/Reviews in Mutation Research*. 1999/07/01/ 1999;437(1):21-49. doi:https://doi.org/10.1016/S1383-5742(99)00037-X

Wei B, O'Connor RJ, Goniewicz ML, Hyland A. Emerging chemicals of health concern in electronic nicotine delivery systems. *Chem Res Toxicol*. Oct 19 2020;33(10):2637-2646. doi:10.1021/acs.chemrestox.0c00281

Westmoreland C, Bender HJ, Doe JE, Jacobs MN, Kass GEN, Madia F, Mahony C, Manou I, Maxwell G, Prieto P, Roggeband R, Sobanski T, Schütte K, Worth AP, Zvonar Z, Cronin MTD. Use of new approach methodologies (nams) in regulatory decisions for chemical safety: Report from an epaa deep dive

workshop. *Regulatory Toxicology and Pharmacology*. 2022/11/01/ 2022;135:105261. doi:https://doi.org/10.1016/j.yrtph.2022.105261

WHO. *Principles and methods for the risk assessment of chemicals in food*. WHO EHC. 2009. EHC 240. https://www.who.int/publications/i/item/9789241572408

Zeiger E. Identification of rodent carcinogens and noncarcinogens using genetic toxicity tests: Premises, promises, and performance. *Regulatory Toxicology and Pharmacology*. 1998/10/01/ 1998;28(2):85-95. doi:https://doi.org/10.1006/rtph.1998.1234